Attorney Docket No. 5051-451IP PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re: Li et al. Confirmation No.: 8515
Serial No.: 09/914,020 Art Unit: 1633
Filed: December 31, 2001 Examiner: J. Epps-Ford
For: *Methods and Compositions for Altering Mucus Secretion*

Date: June 7, 2006

Mailstop Amendment
Commissioner for Patents
PO Box 1450
Alexandria, VA 22313-1450

ATTACHMENT C

Declaration Under 37 CFR § 1.132 of Duncan Fraser Rogers

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Atty. Docket No: 5051-451IP

In re patent application of

Yuehua LI *et al.*

Group Art Unit: 1633

Serial No. 09/914,020

Examiner: Janet L. Epps-Ford

Filed: December 31, 2001

For: METHODS AND COMPOSITIONS FOR ALTERING MUCUS SECRETION

DECLARATION UNDER 37 C.F.R. §1.132

I, Duncan Fraser Rogers, declare that:

1. I hold the academic position of Reader in Respiratory Pharmacology and administrative position of Director of Postgraduate Studies (Research) at the National Heart & Lung Institute, Imperial College London, UK. I hold a PhD in Physiology (University of London) and am a Fellow of the Institute of Biology (UK). I have worked in the field of respiratory physiology and associated pulmonary diseases for over 25 years (from 1980 to the present). A copy of my Curriculum Vitae is appended hereto as **Exhibit 1**.

2. I have read and understood the rejections made in the Office Action in the above-captioned application, mailed on December 7, 2005, based on the alleged lack of written description and alleged lack of enablement by the Examiner. Specifically, the Examiner stated that "there is no evidence that the observed reduction in mucus secretion actually correlated into the amelioration of the symptoms of asthma, since the state of the art, see Barnes (2002) and Rogers (2001 and 2003) as described below, calls into question the actual clinical benefit of mucus inhibitory agents for the treatment of respiratory diseases with associated mucus hypersecretion." I am the sole author of the Rogers cited publications, referred to above as "Rogers (2001 and 2003)," and specifically known as *Chronic Obstructive Pulmonary Disease: Pathogenesis to Treatment: Novartis Foundation Symposium 234*, Volume 234, Edited by D. Chadwick and J.A. Goode, Novartis Foundation 2001, pp. 65-83 (hereinafter referred to as "Rogers 2001") and *Pediatric Pulmonology* 36: 178-188 (2003) (hereinafter referred to as

"Rogers 2003"). In response to the recited basis for Examiner's rejections, I provide the following comments and explanation:

3. As author of these two publications, I do not agree with the Examiner's characterization of portions of the text of these publications which she states calls into question the actual clinical benefit of mucus inhibitory agents for the treatment of respiratory diseases accompanied by mucus hypersecretion. Firstly, Rogers 2001 provides, in part, a historical account of the studies of the contribution of mucus to the physiology and clinical symptoms in COPD from the late 1970's into the mid 1990's. In the studies in the early part of this timeframe, the consensus was that chronic airflow obstruction and mucus hypersecretion were largely independent disease processes. However, a number of studies in the late 1980's and 1990's found a positive correlation between mucus production and the decline in airway function (see the middle of page 71 of Rogers 2001). As I noted in this publication, it is my opinion that mucus hypersecretion clearly contributes to morbidity and mortality in certain groups of patients with COPD (for example in older patents and/or patients who are prone to chest infections and/or patients with low lung function), which is why I suggested that it was important to develop drugs that inhibit mucus hypersecretion in these patients (see pages 72 and 75). When I stated that there was controversy in the pathophysiology and clinical significance of mucus hypersecretion in COPD and the therapeutic value of drugs affecting mucus production, I meant that there was a discrepancy between the data from the earlier 'set' of epidemiological studies compared with the later 'set' of studies. The precise reasons for the discrepancies are not clear but, as stated in the publication (see foot of page 71 and top of page 72), they relate to differences in sampling of occupational cohorts *vs* general populations and choice of outcome measures. It is also likely that because, in a number of the studies above, the same populations, now older, were re-assessed, the impact of airway mucus hypersecretion on morbidity and mortality becomes more apparent with age.

Generally, persons skilled in the pulmonary field have either performed experiments or have analyzed peer reviewed scientific publications that provide evidence that the inhibition of excessive mucus secretion or hypersecretion does result in the alleviation of some or all symptoms which are know to characterize respiratory diseases. This is exemplified in current guidelines for clinical management of asthma that mucus plugging of the airways is as much a

cause of airway obstruction and airflow limitation as are bronchoconstriction and pulmonary inflammation (National Institutes of Health. *Global Initiative for Asthma: pocket guide for asthma management and prevention.* Bethesda: National Institutes of Health, National Heart, Lung and Blood Institute, 2002, publication no. 02-3659). **(See Exhibit 2.)** Similarly, the current recognition that airway mucus hypersecretion is a potential risk factor for an accelerated loss of lung function in COPD has prompted the inclusion of recommendations for mucolytic therapy (i.e. use of drugs to 'thin' mucus) in the latest guidelines for clinical management of COPD (National Institute for Clinical Excellence (NICE). *Chronic obstructive pulmonary disease: national clinical guideline on management of chronic obstructive pulmonary disease in adults in primary and secondary care.* Thorax 2004, 59). **(See Exhibit 3.)** This is in contrast to the lack of such recommendations of only five years ago (National Heart, Lung and Blood Institute/WHO. *Global Initiative for Chronic Obstructive Lung Disease.* Publication no. 2701, National Institutes of Health, 2001), **(See Exhibit 4)** and is testament to our rapidly changing view of the role of mucus hypersecretion in the pathophysiology of COPD. It is important to note here that current treatment of COPD (namely bronchodilators and corticosteroids) is poorly effective (Global Initiative for COPD, 2001 – above). This is in contrast to asthma where these treatments are, for the most part, highly effective. Even the effectiveness of mucolytics is hindered by the lack of potency of current mucolytic compounds. Consequently, there is an unmet clinical need for new therapeutic interventions in COPD and any therapy with potential for alleviating symptoms and disease progression, even by a small amount, would be a valuable asset to clinical management of COPD. Clearly, treatments having beneficial effects on mucus would be part of any pharmacotherapeutic strategy in COPD. The possibility of inhibiting mucus output is an attractive option that would be expected to have therapeutic benefit in patients with COPD in whom mucus hypersecretion contributes significantly to clinical symptoms (e.g. the elderly, patients prone to chest infections and patients with low lung function – see above).

In addition to COPD, as indicated above, mucus hypersecretion is also a clinical feature of severe respiratory conditions in addition to COPD, including asthma, cystic fibrosis (CF) and bronchiectasis. Most patients with asthma cough up sputum (indicative of mucus hypersecretion) at some stage of an asthma attack. This, on top of having to cope with bronchoconstriction and associated breathlessness of an asthma attack, is distressing, tiring and embarrassing for these patients. Excessive luminal mucus in asthma also contributes to airway obstruction and bronchial

hyperresponsiveness (one of the clinical hallmarks of asthma) (Rogers DF. *Airway mucus hypersecretion in asthma: an undervalued pathology?* Current Opinion in Pharmacology 2004, 4: 241-250). (**See Exhibit 5.**) Although bronchodilators and glucocorticosteroids are, for the most part, effective in clinical management of asthma, there are many patients, for example those who are 'steroid insensitive' or have an aberrant polymorphism in their β_2-adrenoceptors that reduces the effectiveness of β_2-agonist bronchodilator therapy. In addition, despite treatment, many patients die in *status asthmaticus*, an end-stage condition of asthma in which the lungs become completely blocked with viscid mucus plugs. Thus, as in COPD, there is an unmet need for additional pharmacotherapy in asthma. Inhibition of excessive airway mucus would, therefore, be expected to alleviate a number of important symptoms of asthma, including cough, sputum production, airway obstruction, bronchial hyperreactivity and mucus plug formation in advanced, severe asthma. CF and bronchiectasis both have airway mucus hypersecretion and recurrent infection of this mucus, associated with disease exacerbation, as clinical features. Consequently, reduction in airway mucus would be expected to limit chest infections and increase quality of life in these vulnerable patient groups.

From the above, it may be seen that there are specific and identifiable reasons why I view inhibition of airway mucus hypersecretion as a valid therapeutic target in both COPD (Rogers 2003, page 184) and asthma (Rogers, 2004). I see anti-MARCKS therapy as a logical and legitimate approach to reducing airway mucus secretion in these conditions. It is also feasible that anti-MARCKS therapy would have clinical benefit in CF and bronchiectasis. It is noteworthy that even though Barnes (2002) states in the abstract that clinical benefits from inhibiting mucus hypersecretion are still not certain, Barnes (2002) also identifies MARCKS inhibitors as potential treatment for airway mucus hypersecretion in COPD, and possibly from multiple causes (pages 245 – 246). The Rogers (2003, 2004) and Barnes (2002) articles cite the high-impact factor journal paper by Li *et al* 2002 (with Adler as senior author - **See Exhibit 6.**) as evidence for their assertion that anti-MARCKS therapy for airway mucus hypersecretion merits consideration. A subsequent paper from Adler and colleagues (Nature Medicine 2004, 10, 193-196) (**See Exhibit 7.**) has extended the original observations, and confirms that Rogers and Barnes were correct in supporting a role for anti-MARCLS therapy for airway mucus hypersecretion.

4. Additionally, the Examiner has raised my disclosure in Rogers 2003 as being supportive of her position of lack of written description and lack of enablement by citing how current treatments for diseases associated with airway mucus hypersecretion do not take into account the homeostatic role of pulmonary mucus and its impact on respiratory physiology. By way of clarification, it is my opinion that it is understood by researchers and physicians within the pulmonary field that a normal level of mucus secretion is important for entrapment of inhaled particles in the mucus gel layer and removal from the airways by mucociliary clearance which provide protective benefits (see Rogers 2003, page 179). However, it is also clear that airway mucus hypersecretion is detrimental to patient health and needs to be treated. Therefore, I believe that persons skilled in the field would understand that mucus inhibition treatment should not inhibit all mucus secretion but rather only inhibit mucus hypersecretion that is excessive and which results in pathophysiological effects on the airway. The correct dosage of drug would be determined in appropriate clinical trials, initially in Phase I trials, and subsequently becoming more refined for clinical use in Phase II and Phase III trials. This procedure holds true for any potential new drug for human use, not just drugs for anti-mucus therapy in the airways. Based upon these trials, skilled persons would be able to determine an appropriate dosage of mucus inhibitor to administer to persons with respiratory diseases that are characterized by mucus hypersecretion.

5. Finally, Professor Barnes and I have just published a joint review article entitled *Treatment of Airway Mucus Hypersecretion* (Annals of Medicine 2006, 38, 116-125) (**See Exhibit 8**). In this article we specifically state that 'long-term and excessive mucus production ... can lead to significant airflow limitation in a number of severe respiratory conditions including asthma, chronic obstructive pulmonary disease (COPD) and cystic fibrosis (CF).' (page 116). We reiterate that there are specific groups of patients in whom airway mucus hypersecretion contributes significantly to morbidity and mortality. We also identify inhibition of mucin exocytosis as a legitimate therapeutic target, with specific mention of anti-MARCKS therapy as a possible pharmacotherapeutic option (page 122). Thus, Professor Barnes and I are in agreement that inhibition of airway mucus hypersecretion is an unmet clinical need for many patients, especially in COPD, but also in asthma, CF and bronchiectasis, and that anti-MARCKS drugs merit consideration as potential anti-hypersecretory therapy.

6. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further, that these statements are made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

5th June 2006
Date

Duncan F. Rogers, Ph.D.

277786 v1/RE

Attorney Docket No. 5051-451IP PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re: Li et al. Confirmation No.: 8515
Serial No.: 09/914,020 Art Unit: 1633
Filed: December 31, 2001 Examiner: J. Epps-Ford
For: *Methods and Compositions for Altering Mucus Secretion*

Date: June 7, 2006

Mailstop Amendment
Commissioner for Patents
PO Box 1450
Alexandria, VA 22313-1450

ATTACHMENT C

Exhibit 1

Curriculum Vitae, Dr. Duncan Fraser Rogers

CURRICULUM VITAE

Full Name and Title: Dr Duncan Fraser ROGERS

Address: Section of Airway Disease
National Heart & Lung Institute
Imperial College London
Dovehouse Street
London SW3 6LY
U.K.

Title of Current Appointment: Reader in Respiratory Pharmacology (1999)
Recognised Teacher of The University of London (Physiology) (1989)

Higher education:

- B.Sc. in Applied Biology (2:1 Hons.), 1976. Brunel University.
- Postgraduate Certificate in Education (P.G. Cert. Ed.), 1977. Garnett College (University of London).
- Ph.D. in Physiology, 1984. University of London.

Membership of Professional Bodies and Learned Societies:

- Membership of the Institute of Biology (M.I.Biol.) 1981, and Chartered Biologist (C.Biol.) 1995.
- Fellow of the Institute of Biology (FIBiol) 2002.
- British Association for Lung Research, 1982.
  Meetings Secretary 1990 -1993
  Chairman, 1993 - 1995.
- British Pharmacological Society, 1988.
- The Physiological Society, 1993.
- Airway Mucus Club, 1986.
  Meetings Secretary 1988-1992.
- British Inflammation Research Association (BIRAS), 1994.
- Member of the British Lung Foundation's Scientific Committee (2000-2003).

Journal Editorships

- *Thorax*: Associate Editor (1995-1997). Member of the Advisory Board (1997-2002)
- *British Journal of Pharmacology*: Member of Editorial Board (1997-2000).
- *Current Drugs*: Member of Panel of Evaluators (1998-present).

- *Experimental Lung Research*: Editor-in-Chief (2001-present).

Publications
Peer-reviewed papers

1. Doig, R.I., Done, A.A. & **Rogers D.F.** (1975) Pre-harvest sprouting in bread wheat (*Triticum aestivum*) as influenced by cytoplasmic male-sterility derived from *T. timopheevi*. Euphytica 24, 229-232.

2. **Rogers, D.F.** & Jeffery, P.K. (1986) Inhibition by oral N-acetylcysteine of cigarette smoke-induced 'bronchitis' in the rat. Exp. Lung Res. 10, 267-283.

3. **Rogers, D.F.** & Jeffery, P.K. (1986) Inhibition of cigarette smoke-induced airway secretory cell hyperplasia by indomethacin, dexamethasone, prednisolone, or hydrocortisone in the rat. Exp. Lung Res. 10, 285-298.

4. **Rogers, D.F.** & Jeffery, P.K. (1986) Indomethacin and flurbiprofen speed recovery of rat bronchial epithelium after exposure to cigarette smoke. Exp. Lung Res. 10, 299-312.

5. **Rogers, D.F.**, Williams, D.A. & Jeffery, P.K. (1986) Nicotine does not cause 'bronchitis' in the rat. Clin. Sci. 70, 427-433.

6. Evans, T.W., Chung, K.F., **Rogers, D.F.** & Barnes, P.J. (1987) Effect of platelet-activating factor on airway vascular permeability: possible mechanisms. J. Appl. Physiol. 63, 479-484.

7. **Rogers, D.F.**, Turner, N.C., Marriott, C. & Jeffery, P.K. (1987) Cigarette smoke-induced 'chronic bronchitis': a study in situ of laryngo-tracheal hypersecretion in the rat. Clin. Sci. 72, 629-637.

8. **Rogers, D.F.**, Godfrey, R.W.A., Majumdar, S. & Jeffery, P.K. (1988) Oral N-acetylcysteine speeds reversal of cigarette smoke-induced mucous cell hyperplasia in the rat. Exp. Lung Res. 14, 19-35.

9. Evans, T.W., Dent, G., **Rogers, D.F.**, Aursudkij, B., Chung, K.F. & Barnes, P.J. (1988) Effect of a PAF antagonist, WEB 2086, on airway microvascular leakage in the guinea-pig and platelet aggregation in man. Br. J. Pharmacol. 94, 164-168.

10. Evans, T.W., **Rogers, D.F.**, Aursudkij, B., Chung, K.F. & Barnes, P.J. (1988) Inflammatory mediators involved in antigen-induced airway microvascular leakage in guinea-pigs. Am. Rev. Respir. Dis. 138, 395-399.

11. **Rogers, D.F.**, Belvisi, M.G., Aursudkij, B., Evans, T.W. & Barnes, P.J. (1988) Effects and interactions of sensory neuropeptides on airway microvascular leakage in

guinea pigs. Br. J. Pharmacol. 95, 1109-1116.

12. Boschetto, P., Roberts, N.M., **Rogers, D.F.** & Barnes, P.J. (1989) Effect of anti-asthma drugs on microvascular leakage in guinea-pig airways. Am. Rev. Respir. Dis. 139, 416-421.

13. Belvisi, M.G., **Rogers, D.F.** & Barnes, P.J. (1989) Neurogenic plasma extravasation: inhibition by morphine in guinea pig airways in vivo. J. Appl. Physiol. 66, 268-272.

14. Evans, T.W., **Rogers, D.F.**, Aursudkij, B., Chung, K.F. & Barnes, P.J. (1989) Regional and time-dependent effects of inflammatory mediators on airway microvascular permeability in the guinea pig. Clin. Sci. 76, 479-485.

15. **Rogers, D.F.** & Barnes, P.J. (1989) Opioid inhibition of neurally mediated mucus secretion in human bronchi. The Lancet i, 930-932.

16. **Rogers, D.F.**, Boschetto, P. & Barnes, P.J. (1989) Plasma exudation: correlation between Evans blue dye and radiolabelled albumin in guinea pig airways in vivo. J. Pharmacol. Methods 21, 309-315.

17. **Rogers, D.F.**, Turner, N.C., Marriott, C. & Jeffery, P.K. (1989) Oral N-acetylcysteine or S-carboxymethylcysteine inhibit cigarette smoke-induced hypersecretion of mucus in rat larynx and trachea in situ. Eur. Resp. J. 2, 955-960.

18. **Rogers, D.F.**, Aursudkij, B. & Barnes, P.J. (1989) Effects of tachykinins on mucus secretion in human bronchi in vitro. Eur. J. Pharmacol. 174, 283-286.

19. Evans, T.W., **Rogers, D.F.**, Belvisi, M.G., Rohde, J.A.L., Chung, K.F. & Barnes, P.J. (1990) Endotoxin-induced plasma exudation in guinea pig airways in vivo and the effect of neutrophil depletion. Eur. Resp. J. 3, 299-303.

20. Evans, T.W., McAnulty, R.J., **Rogers, D.F.**, Chung, K.F., Barnes, P.J. & Laurent, G.J. (1990) Bleomycin-induced lung injury in the rat: effects of the platelet activating factor (Paf) receptor antagonist BN 52021 and platelet depletion. Env. Health Perspectives 85, 65-69.

21. Belvisi, M.B., Barnes, P.J. & **Rogers, D.F.** (1990) Neurogenic inflammation in the airways: characterisation of electrical parameters for vagus nerve stimulation in the guinea-pig. J. Neurosci. Methods 32, 159-167.

22. Tokuyama, K., Kuo, H.-P., Rohde, J.A.L., Barnes, P.J. & **Rogers, D.F.** (1990) Neural control of goblet cell secretion in guinea pig airways. Am. J. Physiol. 259, L108-L115.

23. **Rogers, D.F.**, Alton, E.W.F.W., Aursudkij, B., Boschetto, P., Dewar, A. & Barnes, P.J. (1990) Effect of platelet activating factor on formation and composition of airway fluid in the guinea-pig. J. Physiol. 431, 643-658.

24. **Rogers, D.F.**, Dijk, S. & Barnes, P.J. (1990) Bradykinin-induced plasma exudation in guinea pig airways: involvement of platelet activating factor. Br. J. Pharmacol. 101, 739-745.

25. Kuo, H.-P., Rohde, J.A.L., Tokuyama, K., Barnes, P.J. & **Rogers, D.F.** (1990) Capsaicin and sensory neuropeptide stimulation of goblet cell secretion in guinea-pig trachea. J. Physiol. 431, 629-641.

26. **Rogers, D.F.**, Alton, E.W.F.W., Dewar, A., Geddes, D.M. & Barnes, P.J. (1990) Tracheal potential difference in the reserpine and isoproterenol rat models of cystic fibrosis. Exp. Lung Res. 16, 661-670.

27. Boschetto, P., **Rogers, D.F.**, Fabbri, L.M. & Barnes, P.J. (1991) Corticosteroid inhibition of airway microvascular leakage. Am. Rev. Respir. Dis. 143, 605-609.

28. **Rogers, D.F.**, Godfrey, R.W.A., Castro, K., & P.K. Jeffery (1991) Effects of a new compound (Zy 15850A) on cigarette smoke-induced bronchitis in the rat. Agents Actions 33, 359366.

29. Lei, Y.-H., Barnes, P.J. & **Rogers, D.F.** (1992) Inhibition of neurogenic plasma exudation in guinea pig airways by CP-96,345, a new non-peptide NK_1 antagonist. Br. J. Pharmacol. 105, 261-262 (*'Special Report'*).

30. Kuo, H.-P., Rohde, J.A.L., Barnes, P.J. & **Rogers D.F.** (1992) Differential inhibitory effects of opioids on cigarette smoke, capsaicin and electrically-induced goblet cell secretion. Br. J. Pharmacol. 105, 361-366.

31. Kuo, H.-P., Rohde, J.A.L., Barnes, P.J. & **Rogers, D.F.** (1992) K^+ channel activator inhibition of neurogenic goblet cell secretion in guinea pig trachea. Eur. J. Pharmacol. 215, 297-299.

32. Kuo, H.-P., Rohde, J.A.L., Barnes, P.J. & **Rogers, D.F.** (1992) Cigarette smoke-induced airway goblet cell secretion: dose-dependent differential nerve activation. Am. J. Physiol. 263, L161-167.

33. Alton, E.W.F.W., **Rogers, D.F.**, Logan-Sinclair, R., Yacoub, M., Barnes, P.J. & Geddes, D.M. (1992). Bioelectric properties of cystic fibrosis airways obtained from heart-lung transplantation. Thorax 47, 1010-1014.

34. Hui, K.P., Lotvall, J., **Rogers, D.F.**, Barnes, P.J. & Chung, K.F. (1992) Ovalbumin aerosol challenge in actively sensitized guinea pigs: relationship between

airway microvascular leakage and airflow obstruction. Allergy 47, 527-531.

35. Lei, Y.-H., Barnes, P.J. & **Rogers, D.F.** (1993) Regulation of NANC neural bronchoconstriction *in vivo* in the guinea pig: involvement of nitric oxide, vasoactive intestinal peptide and soluble guanylyl cyclase. Br. J. Pharmacol. 108, 228-235.

36. **Rogers, D.F.**, Alton, E.W.F.W., Dewar, A., Lethem, M.I. & Barnes, P.J. (1993) Impaired stimulus-evoked mucus secretion in cystic fibrosis bronchi. Exp. Lung Res. 19, 37-53.

37. Hirayama, Y., Lei, Y.-H., Barnes, P.J. & **Rogers, D.F.** (1993) Effects of two novel tachykinin antagonists, FK224 and FK888, on neurogenic airway plasma exudation, bronchoconstriction and systemic hypotension in guinea-pigs *in vivo*. Br. J. Pharmacol. 108, 844-851.

38. Meini, S., Mak, J.C.W., Rohde, J.A.L. & **Rogers, D.F.** (1993) Tachykinin control of ferret airways: mucus secretion, bronchoconstriction and receptor mapping. Neuropeptides 24, 81-89.

39. Lei, Y.-H., Barnes, P.J. & **Rogers, D.F.** (1993) Inhibition of neurogenic plasma exudation and bronchoconstriction by a K^+ channel activator, BRL 38227, in guinea pig airways *in vivo*. Eur. J. Pharmacol. 239, 257-259.

40. Ramnarine, S.I., Hirayama, Y., Barnes, P.J. & **Rogers, D.F.** (1994) 'Sensory-efferent' neural control of mucus secretion: characterisation using tachykinin receptor antagonists in ferret trachea *in vitro*. Br. J. Pharmacol. 113, 1183-1190.

41. Hayes, J.P., Kuo, H.-P., Rohde, J.A.L., Newman Taylor, A.J., Barnes, P.J., Chung, K.F. & **Rogers, D.F.** (1995) Neurogenic goblet cell secretion and bronchoconstriction in guinea pigs sensitised to trimellitic anhydride. Eur. J. Pharmacol. Environ. Tox. Pharmacol. Section 292, 127-134.

42. Lei, Y.-H., Barnes, P.J. & **Rogers, D.F.** (1995) Mechanisms and modulation of airway plasma exudation after direct inhalation of cigarette smoke. Am. J. Respir. Crit. Care Med. 151, 1752-1762.

43. Lei, Y.-H., Barnes, P.J. & **Rogers, D.F.** (1996) Involvement of hydroxyl radicals in neurogenic airway plasma exudation and bronchoconstriction in guinea-pigs *in vivo*. Br. J. Pharmacol. 117, 449-454.

44. Ramnarine, S.I., Khawaja, A.M., Barnes, P.J. & **Rogers, D.F.** (1996) Nitric oxide inhibition of basal and neurogenic mucus secretion in ferret trachea *in vitro*. Br J Pharmacol. 118, 998-1002.

45. Ramnarine, S.I., Haddad, E.-B., Khawaja, A.M., Mak, J.C.W. & **Rogers, D.F.**

(1996) On muscarinic control of neurogenic mucus secretion in ferret trachea. J. Physiol. 494, 577-586.

46. Newman, T.M., Robichaud, A. & **Rogers, D.F.** (1996) Microanatomy of secretory granule release from guinea pig tracheal goblet cells. Am. J. Respir. Cell Mol. Biol. 15: 529-539.

47. Emms, J.C. & **Rogers, D.F.** (1997) Cigarette smoke-inhibition of neurogenic bronchoconstriction in guinea pigs *in vivo*: involvement of exogenous and endogenous nitric oxide. Br. J. Pharmacol. 121: 779-785.

48. Nightingale, J.A., **Rogers, D.F.** & Barnes, P.J. (1998) The effect of repeated sputum induction on cell counts in normal volunteers. Thorax 53: 87-90.

49. Ramnarine, S.I., Liu, Y.-C. & **Rogers, D.F.** (1998) Neuroregulation of mucus secretion by opioid receptors and K_{ATP} and BK_{Ca} channels in ferret trachea *in vitro*. Br. J. Pharmacol. 123: 1631-1638.

50. Liu, Y.-C., Khawaja, A.M. & **Rogers, D.F.** (1998) Effects of the cysteinyl leukotriene receptor antagonists pranlukast and zafirlukast on tracheal mucus secretion in ovalbumin-sensitized guinea pigs *in vitro*. Br. J. Pharmacol. 124: 563-571.

51. Nightingale, J.A., **Rogers, D.F.**, Hart, L.A., Kharitonov, S.A., Chung, K.F. & Barnes, P.J. (1998) Effect of inhaled endotoxin on induced sputum in normal, atopic, and atopic asthmatic subjects. Thorax 53: 563-571.

52. Liu, Y.-C., Patel, H.J., Khawaja, A.M., Belvisi, M.G. & **Rogers, D.F.** (1999) Neuroregulation by vasoactive intestinal peptide (VIP) of mucus secretion in ferret trachea: activation of BK_{Ca} channels and inhibition of neurotransmitter release. Br. J. Pharmacol. 126: 147-158.

53. Lei, Y.-H. & **Rogers, D.F.** (1999) Effects and interactions of opioids on plasma exudation induced by cigarette smoke in guinea pig bronchi. Am. J. Physiol. 276, L391-L397.

54. Nightingale, J.A., **Rogers, D.F.** & Barnes, P.J. (1999) Differential effect of formoterol on adenosine monophosphate and histamine reactivity in asthma. Am. J. Respir. Crit. Care Med. 159, 1786-1790.

55. Liu, Y.-C., Khawaja, A.M. & **Rogers, D.F.** (1999) Effect of vasoactive intestinal peptide (VIP)-related peptides on cholinergic neurogenic and direct mucus secretion in ferret trachea *in vitro*. Br. J. Pharmacol. 128, 1353-1359.

56. Nightingale, J.A., **Rogers, D.F.** & Barnes, P.J. (1999) Effect of inhaled ozone on

exhaled nitric oxide in normal subjects and asthmatic subjects. Thorax 54, 1061-1069.

57. Khawaja, A.M., Liu, Y.-C. & **Rogers, D.F.** (1999) Effect of fenspiride, a non-steroidal antiinflammatory agent, on neurogenic mucus secretion in ferret trachea in vitro. Pulm. Pharmacol. Therapeutics 12, 363-368.

58. Khawaja, A.M., Liu, Y.-C. & **Rogers, D.F.** (1999) Effect of non-peptide tachykinin NK_1 receptor antagonists on non-adrenergic, non-cholinergic neurogenic mucus secretion in ferret trachea. Eur. J. Pharmacol. 384, 173-181.

59. Nightingale, J.A., **Rogers, D.F.**, Chung, K.F. & Barnes, P.J. (2000) No effect of inhaled budesonide on the response to inhaled ozone in normal subjects. Am. J. Respir. Crit. Care Med. 161, 479-486.

60. Nightingale, J.A., Maggs, R., Cullinan, P., Donnelly, L.E., **Rogers, D.F.**, Kinnersley, R., Chung, K.F. & Barnes, P.J., Ashmore, M., Newman-Taylor, A. (2000) Airway inflammation after controlled exposure to diesel exhaust particulates. Am. J. Respir. Crit. Care Med. 162, 161-166.

61. Khan, S., Liu, Y.-C., Khawaja, A.M., Manzini, S. & **Rogers, D.F.** (2001) Effect of the long-acting tachykinin NK1 receptor antagonist MEN 11467 on tracheal mucus secretion in allergic ferrets. Br. J. Pharmacol. 132, 189-196.

62. Nightingale, J.A., **Rogers, D.F.** & Barnes, P.J. (2002) Comparison of the effects of salmeterol and formoterol in patients with severe asthma. Chest 121, 1401-1406.

63. Culpitt, S.V., de Matos, C., Russell, R.E., Donnelly, L.E., **Rogers, D.F.** & Barnes, P.J. (2002) Effect of theophylline on induced sputum inflammatory indices and neutrophil chemotaxis in COPD. Am. J. Respir. Crit. Care Med. 165, 1371-1376.

64. Culpitt, S.V., **Rogers, D.F.**, Shah, P., de Matos, C., Russell, R.E.K., Donnelly, L.E. & Barnes, P.J. (2003) Impaired inhibition by dexamethasone of cytokine release by alveolar macrophages from COPD patients. Am. J. Respir. Crit. Care Med. 167, 24-31.

65. Culpitt, S.V., **Rogers, D.F.**, Fenwick, P.S., Shah, P., de Matos, C., Russell, R.E.K., Barnes, P.J. & Donnelly, L.E. (2003) Inhibition by red wine extract, resveratrol, of cytokine release by alveolar macrophages in COPD. Thorax 58, 942-946.

66. Culpitt, S.V., **Rogers, D.F.**, Traves, S.L., Barnes, P.J. & Donnelly, L.E. (2005) Sputum matrix metalloproteases: comparison between chronic obstructive pulmonary disease and asthma. Respir. Med. 99, 703-710.

Reviews/articles

1. Jeffery, P.K., **Rogers, D.F.** & Ayers, M. (1985) Effect of oral acetylcysteine on tobacco smoke-induced secretory cell hyperplasia. Eur. J. Respir. Dis. 66 (suppl. 139), 117-122.

2. Barnes, P.J., Belvisi, M.G. & **Rogers, D.F.** (1990) Modulation of neurogenic inflammation: novel approaches to inflammatory diseases. Trends Pharmacol. Sci. 11, 185-189.

3. **Rogers, D.F.** & Dewar, A. (1990) Neural control of airway mucus secretion. Biomedicine & Pharmacotherapy 44, 447-453.

4. **Rogers, D.F.** & Evans, T.W. (1992) Plasma exudation and oedema: major contributors to asthma? British Medicial Bulletin 48, 120-134.

5. Ramnarine, S.I. & **Rogers, D.F.** (1994) Non-adrenergic, non-cholinergic neural control of mucus secretion in the airways. Pulmonary Pharmacol. 7, 19-33.

6. **Rogers, D.F.** (1994) Airway goblet cells: responsive and adaptable front line defenders. Eur. Respir. J. 7, 1690-1706.

7. **Rogers, D.F.** & Ganderton, D. (1995) Determining equivalence of inhaled medications. Respir. Med. 89, 253-261.

8. **Rogers, D.F.** (1995) Neurokinin receptors subserving airways secretion. Canadian J. Physiol. Pharmacol. 73, 932-939.

9. Tetley, T.D. & **Rogers, D.F.** (1996) Development of new treatments for lung disease. Respir. Med. 90, 5-23.

10. **Rogers, D.F.** (1996) Scorpion venoms: taking the sting out of lung disease. Thorax 51, 546-548.

11. Khawaja, A.M. & **Rogers, D.F.** (1996) Tachykinins: receptor to effector. International J. Biochem. Cell Biol. 28, 721-738.

12. **Rogers, D.F.** (1997) Neurogenic inflammation in lung disease: burnt out? Inflammopharmacology 5, 319-329.

13. **Rogers, D.F.** (1997) *In vivo* test models for studying airway mucus secretion. Pulmonary Pharmacol. Therapeutics 10, 121-128.

14. **Rogers, D.F.** & Laurent, G.J. (1998) New ideas on pathophysiology and treatment of lung disease. Thorax 53, 200-210.

15. **Rogers, D.F.** & Giembycz, M.A. (1998) Asthma therapy for the 21st Century.

Trends Pharmacol. Sci. 19, 160-164.

16. **Rogers, D.F.** & Giembycz, M.A. (1998) Conquering airway inflammation in the 21st Century. Drug Discovery Today 3, 532-535.

17. **Rogers, D.F.** & Barnes, P.J. (1999) COPD: New developments and therapeutic opportunities. Trends Pharmacol. Sci. 20, 352-354.

18. Matthews, J.G. & **Rogers, D.F.** (1999) rhuMab-E25. Curr. Opin. Anti-inflamm. Immunomodulatory Investigational Drugs 1, 454-461.

19. Culpitt, S. & **Rogers, D.F.** (2000) Evaluation of current pharmacotherapy of chronic obstructive pulmonary disease. Expert Opinion Pharmacother. 1, 1007-1020.

20. **Rogers, D.F.** (2000) Mucus pathophysiology in COPD: differences to asthma, and pharmacotherapy. Monaldi Arch. Chest Dis. 55, 324-332.

21. **Rogers, D.F.** (2000) Motor control of airway goblet cells and glands. Respir. Physiol. 125 , 129-144.

22. **Rogers, D.F.** (2001) Tachykinin receptor antagonists for asthma and COPD. Exp. Opinion Ther. Patents 11, 1097-1121.

23. **Rogers, D.F.** (2002) Mucoactive drugs for asthma and COPD: any place in therapy? Exp. Opinion Invest. Drugs 11, 15-35.

24. **Rogers, D.F.** (2002) Pharmacological regulation of the neuronal control of airway mucus secretion. Curr. Opin. Pharmacol. 2, 249-255.

25. Pritchard, K., Smith, A.K. & **Rogers, D.F.** (2002) Measuring mucin gene expression in human airways - Northern analysis and RT-PCR. Thorac. Med. 17, 1-9.

26. **Rogers, D.F.** (2003) The airway goblet cell. Int. J. Biochem. Cell Biol. 35, 1-6.

27. **Rogers, D.F.** (2003) Airway hypersecretion in allergic rhinitis and asthma: new pharmacotherapy. Curr. Allergy Asthma Reports 3, 238-248.

28. **Rogers, D.F.** (2003) Pulmonary mucus: pediatric perspective. Pediatric Pulmonol. 36, 178-188.

29. Donnelly, L.E. & **Rogers, D.F.** (2003) Therapy for chronic obstructive pulmonary disease in the 21st Century. Drugs 63, 1973-1998.

30. Donnelly, L.E. & **Rogers, D.F.** (2003) Antiproteases and retinoids for treatment of chronic obstructive pulmonary disease. Exp Opinion Therapeutic Drugs 13, 1345-

1372.

31. **Rogers, D.F.** (2004) Airway mucus hypersecretion in asthma: an undervalued pathology? Curr. Opinion Pharmacol. 4, 241-250.

32. **Rogers, D.F.** (2005) Mucociliary dysfunction in COPD: effect of current pharmacotherapeutic options. Pulm. Pharmacol. Ther. 18, 1-8.

33. **Rogers, D.F.** (2005) The role of airway secretions in COPD: pathophysiology, epidemiology and pharmacotherapeutic options. COPD: J. Chronic Obstructive Pulmonary Disease 2, 341-353.

34. **Rogers, D.F.** & Barnes, P.J. (2006) Treatment of airway mucus hypersecretion. Ann. Med. 38, 116-125.

35. **Rogers, D.F.** (2006) Airway mucus hypersecretion: rationales for pharmacotherapy. J. Organ Dysfunctions (in press).

36. Foster, K., Adams, E.J., Durose, L., Cruttwell, C.J., Marks, E., Shone, C.C., Chaddock, J.A., Cox, C.L., Heaton, C., Sutton, J.M., Wayne, J., Alexander, F.C.G. & **Rogers, D.F.** (2006) Re-engineering the target specificity of clostridial neurotoxins - a route to novel therapeutics. Neurotoxicity Research (in press).

Book chapters

1. Jeffery, P.K., Ayers, M. & **Rogers, D.F.** (1982) The mechanisms and control of bronchial mucous cell hyperplasia. *In*: Mucus in Health and Disease II, eds. E.N. Chantler, J.B. Elder & M. Elstein, Adv. Exp. Med. Biol. pp. 144, 399-409. Plenum Press, London.

2. Jeffery, P.K., **Rogers, D.F.**, Ayers, M., Evans, P.M. & Williams, D.A. (1984) Chronic effects of drugs on airway mucus-secreting cells. *In*: Drugs and the Lung, eds. G. Cumming & G. Bonsignore, Life Science Series 14, pp. 87-115. Plenum Publishing Corporation, London.

3. Jeffery, P.K., **Rogers, D.F.**, Ayers, M. & Shields, P.A. (1984) Structural aspects of cigarette smoke-induced pulmonary disease. *In*: Smoking and the Lung, eds. G. Cumming & G. Bonsignore, Life Science Series, pp. 1-31. Plenum Publishing Corporation, London.

4. **Rogers, D.F.**, Alton, E.W.F.W. & Barnes, P.J. (1990) Airway secretion. *In*: The Metabolic and Molecular Basis of Acquired Disease, eds. R.D. Cohen, K.G.M.M. Alberti, B. Lewis & A.M. Denman, vol. 2, pp. 1979-2010. Balliere Tindall, London.

5. **Rogers, D.F.** (1993) Mucous glands and goblet cells. *In*: Pharmacology of the

Respiratory Tract: Clinical and Experimental, eds. K.F. Chung & P.J. Barnes. Lung Biology in Health and Disease, ed. C. Lenfant, pp 583-620. Marcel Dekker Inc., New York.

6. **Rogers, D.F.** (1994) Influence of respiratory tract fluid on airway calibre. *In*: Airways Smooth Muscle: Development, and Regulation of Contractility, eds. D. Raeburn & M.A. Giembycz, pp. 375-409. Birkhäuser Verlag, Basel, Switzerland.

7. **Rogers, D.F.** (1997) Neural control of airway secretions. *In*: The Autonomic Nervous System, ed. G. Burnstock, vol. 7, Autonomic Control of the Respiratory System, ed. P.J. Barnes, pp. 201-227. Harwood Academic Publishers GmbH, The Netherlands.

8. **Rogers, D.F.** & Barnes, P.J. (1997) Neural control of the airway vasculature. *In*: The Autonomic Nervous System, ed. G. Burnstock, vol. 7, Autonomic Control of the Respiratory System, ed. P.J. Barnes, pp. 229-248. Harwood Academic Publishers GmbH, The Netherlands.

9. Newman, T.M. & **Rogers, D.F.** (1997) The microanatomy of airway mucus secretion. *In*: Airway Mucus: Basic Mechanisms and Clinical Perspectives, eds. D.F. Rogers & M.I. Lethem, pp. 67-89. Birkhäuser Verlag, Basel, Switzerland.

10. Fung, D.C.K. & **Rogers, D.F.** (1997) Airway submucosal glands: physiology and pharmacology. *In*: Airway Mucus: Basic Mechanisms and Clinical Perspectives, eds. D.F. Rogers & M.I. Lethem, pp. 179-210. Birkhäuser Verlag, Basel, Switzerland.

11. Liu, Y.C., Khawaja, A.M. & **Rogers, D.F.** (1998) Pathophysiology of airway mucus secretion in asthma. *In*: Asthma: Basic Mechanisms and Clinical Management, Third Edition, eds. P.J. Barnes, I.W. Rodger & N.C. Thomson, pp. 205-227. Academic Press, London.

12. Belvisi, MG. & **Rogers, D.F.** (1998) Neurogenic inflammation in the airways: measurement of microvascular leakage. *In*: Methods in Pulmonary Research, eds. S. Uhlig & A.E. Taylor, pp. 231-250. Birkhäuser Verlag, Basel, Switzerland.

13. **Rogers, D.F.** (2001) Mucus hypersecretion in chronic obstructive pulmonary disease. *In*: Chronic Obstructive Pulmonary Disease: Pathogenesis to Treatment. Novartis Foundation Symposium 234, pp. 65-83. John Wiley & Sons, Ltd, Chichester.

14. **Rogers, D.F.** (2001) Mucus regulation. *In*: New Drugs for Asthma, Allergy and COPD, eds. T.T. Hansel & P.J. Barnes. Progress in Respiratory Research, vol 31, pp 160-164. Karger, Basel.

15. **Rogers, D.F.** (2001) Muscarinic control of airway mucus secretion. *In*:

Muscarinic Receptors in Airway Diseases, eds. J. Zaagsma, H. Meurs, A.F. Roffel, pp. 175-201. Birkhäuser Verlag, Basel, Switzerland.

16. Pritchard, K., Smith, A.K. & **Rogers, D.F.** (2001) Measurement of airway mucin gene expression. *In*: Human Airway Inflammation: Sampling Techniques and Analytical Protocols, eds. Rogers, D.F. & Donnelly, L.E. Methods in Molecular Medicine series, pp. 285-294. Humana Press Inc., Totowa, U.S.A.

17. Smith, A.K. & **Rogers, D.F.** (2001) In vivo models of airway goblet cell hyperplasia and mucin gene expression. *In*: Cilia and Mucus: From Development to Respiratory Defense, ed. M. Salathe, pp. 239-251. Marcel Dekker Inc., New York.

18. Nightingale, J.A. & **Rogers, D.F.** (2002) Should drugs affecting mucus properties be used in stable COPD? Clinical Evidence. *In*: Clinical Management of Stable COPD, eds. T. Similowski, W.A. Whitelaw & J.-P. Derenne. Lung Biology in Health and Disease, ed. C. Lenfant, pp. 405-425. Marcel Dekker Inc., New York.

19. **Rogers, D.F.** (2004) Mucus hypersecretion in COPD. *In*: Recent Advances in Pathophysiology of COPD, eds. T.T. Hansel & P.J. Barnes. Progress in Inflammation Research, ed. M.J. Parnham, pp. 101-119. Birkhäuser Verlag, Basel, Switzerland.

20. **Rogers, D.F.** (2004) Overview of airway mucus clearance. *In*: Therapy of Mucus Clearance Disorders, eds. B. Rubin & C. van der Schans, pp. 1-27. Marcel Dekker Inc., New York.

21. **Rogers, D.F.** (2005) Airway mucus in COPD: pathophysiology and treatment. *In*: Chronic Obstructive Pulmonary Disease: Cellular and Molecular Mechanisms, ed. P.J. Barnes, pp. 83-111. New York, Marcel Dekker.

22. **Rogers, D.F.** & Rubin, B.K. (2006) Mucolytics for COPD. *In*: COPD, eds. R. Stockley, S. Rennard, K. Rabe & Celi, B. Blackwell, Oxford, UK (in press).

Editorials

1. **Rogers, D.F.** & O'Connor, B.J. (1993) Airway hyperresponsiveness: relation to asthma and inflammation? Thorax 48, 1095-1096.

2. **Rogers, D.F.** (1996) Reflexly runny noses: neurogenic inflammation in the nasal mucosa. Clin. Exp. Allergy 26, 365-367.

3. **Rogers, D.F.** (2002) Airway goblet cell hyperplasia in asthma: hypersecretory and antiinflammatory? Clin. Exp. Allergy 32, 1124-1127.

Other articles

1. **Rogers, D.F.** (1986) Oralt acetylcystein hammar "bronkit" hos ratta. Tika Information (AB Tika, Lund, Sweden) 6, 1-4.

2. **Rogers, D.F.** (1988) Cystisk pankreasfibros - rapport fran en orskningskonferens. Tika Information (AB Tika, Lund, Sweden) 1, 2-4.

3. **Rogers, D.F.** (1996) Breathing new life into asthma treatments. Biologist (Journal of the Institute of Biology) 43, 81-85.

Electronic publications

1. Nightingale, J.A. & **Rogers D.F.** (1999) Evaluation of R,R-formoterol. Current Drugs Ltd., Investigational Drugs Database: http://www.IDdb.com (13th January).

2. **Rogers, D.F.** (1999) Evaluation of SKF-94120. Current Drugs Ltd., Investigational Drugs Database: http://www.IDdb.com (18th January).

Editor, Review Series

1. **Rogers, D.F.** (1992-1993) New Perspectives on Basic Mechanisms in Lung Disease. Thorax 47 (12) - 48 (5). Introduction: (1992) 47, 1063.

2. Laurent, G.J. & **Rogers, D.F.** (1996-1998) Science Matters. Thorax 51 (2) - 53 (3). Introduction: **Rogers, D.F.** & Laurent, G.J. (1996) 51, 217.

Books

1. **Rogers, D.F.** (1984) The effect of tobacco smoke and nicotine on rat airway epithelium and the response of tobacco smoke-induced secretory cell hyperplasia to anti-inflammatory drugs. Ph.D. Thesis, University of London.

2. **Rogers, D.F.** & Lethem, M.I. eds. (1997) Airway Mucus: Basic Mechanisms and Clinical Perspectives. Respiratory Pharmacology and Pharmacotherapy series. Basel, Switzerland, Birkhäuser Publishing Ltd.

3. **Rogers, D.F.** & Donnelly, L.E. eds. (2001) Human Airway Inflammation: Sampling Techniques and Analytical Protocols. Methods in Molecular Medicine series. Totowa, U.S.A., Humana Press Inc.

Talks/seminars/oral presentations

1981

1. The Canadian Congress of Laboratory Medicine. University of Toronto, Canada. The effect of antiinflammatory agents on the response of bronchial epithelium to

tobacco smoke.

1982

2. British Association for Lung Research, Autumn Meeting. Chelsea College, London. The effect of nicotine on experimental bronchial secretory cell hyperplasia.

3. Biomedical Research Seminar. Cardiothoracic Institute, London. Experimental bronchitis.

1983

4. Pathological Society of Great Britain & Ireland, Winter Meeting. University of Birmingham. Inhibition of tobacco smoke-induced secretory cell hyperplasia in rat airways by anti-inflammatory drugs.

5. British Association for Lung Research, Autumn Meeting. University of Edinburgh. The inhibitory effect of the mucolytic agent, N-acetylcysteine, on experimentally-induced bronchitis.

1984

6. Pathological Society of Great Britain & Ireland, Winter Meeting. Royal Postgraduate Medical School, Hammersmith Hospital, London. The inhibitory effect of the mucolytic agent, N-acetylcysteine, on experimentally-induced bronchitis.

7. Physiological Society, Imperial College Meeting, London. Tobacco smoke-induced tracheal hypersecretion in the rat.

8. European Society for Clinical Investigation. University of Milan, Italy. Experimental hypersecretion of tracheal mucus.

9. Ninth International Cystic Fibrosis Congress. Metropole Hotel, Brighton. Inhibition and reversibility of experimental bronchitis.

10. Medical Research Society. University of Oxford. Effect of oral N-acetylcysteine on tracheal hypersecretion in the rat.

11. Biomedical Research Seminar. Cardiothoracic Institute, London. Modulation of experimental bronchitis.

12. British Association for Lung Research, Summer Meeting. Cardiothoracic Institute, London. Do rats catch C.O.L.D.?

1985

13. Research and Development Seminar, AB Draco, Lund, Sweden. Development of animal models of chronic bronchitis.

14. Physiology Seminar. St. George's Hospital Medical School, London. Mechanisms and modulation of experimental bronchitis.

1986

15. 14th Annual Meeting of the European Working Group for Cystic Fibrosis. Budapest, Hungary. Control of airways' secretion in CF.

16. British Pharmacological Society. The Hatfield Polytechnic. Human bronchial secretion: effect of substance P, muscarinic and adrenergic stimulation in vitro.

1987

17. Airway Mucus Club. Cardiothoracic Institute, London. Airway mucus in CF.

18. Biomedical Research Seminar. Cardiothoracic Institute, London. Abnormal autonomic control of mucus secretion in cystic fibrosis bronchi.

19. British Association for Lung Research, Spring Workshop. Town Hall, Henley-upon-Thames. Use of Evans blue dye to study airway microvascular permeability.

20. The Physiological Society, Mill Hill Meeting, London. Effect of substance P, neurokinins and calcitonin gene-related peptide on microvascular permeability in guinea pig airways.

21. Medical Research Society. Royal Postgraduate Medical School, Hammersmith Hospital Medical School, London. Bradykinin-induced microvascular leakage in guinea pig airways: involvement of platelet activating factor and prostanoids.

1988

22. Airway Mucus Club. St. George's Hospital Medical School, London. Substance P and mucus secretion.

23. Cystic Fibrosis Research Trust: Research Worker's Conference. University of Manchester. Abnormal autonomic control of mucous secretion in CF bronchi in vitro: reduced response to agonist drugs.

24. British Thoracic Society, Autumn Meeting. Kensington Town Hall, London. Inhibition of airway microvascular leakage by corticosteroids.

1989

25. 24th Annual Congress of the SEPCR. Palais de Beaulier, Lausanne, Switzerland. Platelet activating factor affects formation and composition of respiratory tract fluid.

26. XIVth Congress of European Academy of Allergology and Clinical Immunology. West Berlin, FRG. The effect of mediators on microvascular permeability.

1990

27. Respiratory Division Seminar. Hammersmith Hospital, London. Airway mucus secretion.

28. Taunus Medical Society. Frankfurt, FRG. Novel approaches in control of airway mucus.

29. American Thoracic Society. Boston, USA. Effect of platelet activating factor on bioelectric properties of guinea pig trachea in vitro.

30. Joint Meeting SEP-SEPCR. London, UK. Tachykinins and airway secretion.

31. Airway Mucus Club. St. George's Hospital Medical School, London. Neuropeptides and goblet cell secretion.

1991

32. Transatlantic Airway Conference on Airway Mucins, Miami, USA. Invited Discussant.

33. Joint Meeting of the British Pharmacological Society and the Association Francaise des Pharmacologistes, Lyon, France. A potassium channel activator (Lemakalim) modulates vagally-mediated goblet cell secretion in guinea pig trachea.

34. International Union of Physiological Sciences, Prague, Czechoslovakia. Neurogenic inflammation in the airways: mechanisms and modulation.

35. Respiratory Division Seminar, Royal Postgraduate Medical School, Hammersmith Hospital, London. Neurogenic airway secretion.

36. Physiology Department Seminar, St George's Hospital Medical School, London. Airways secretions: mechanisms and modulations.

1992

37. Research Seminar, Roche Products Limited, Welwyn Garden City. Neuropeptides and airway secretion: mechanisms and modulation.

38. Research Seminar, Bayer plc, Stoke Poges. Contributory factors in chronic inflammatory lung obstruction.

39. Research Seminar, Institute du Recherche Jouveinal, Paris, France. Pharmacology of airway secretion.

1993

40. XXXII International Union of Physiological Sciences Congress, Glasgow, Scotland. Goblet cells.

41. Research Seminar, Pfizer Central Research, Sandwich. Muscarinic and tachykinin control of airway function.

42. European Respiratory Society Annual Congress, Florence, Italy. Neuropeptide control of mucus production.

1994

43. Taunus Medical Society, Mannheim, Germany. New therapeutic concepts for asthma.

44. Airway Mucus Club, Kings College London. Tachykinin receptors mediating secretion of airway mucus.

45. Satellite Symposium of the XIIth International Union of Pharmacology (IUPHAR) Congress: Peptides and their Antagonists in Tissue Injury; Montreal, Canada. Neurokinin receptors subserving airways secretion.

1995

46. West London Respiratory Meeting, National Heart & Lung Institute, London. Inhibition of neurogenic airway mucus secretion.

47. Asthma Directorate, Royal Brompton Hospital NHS Trust, London. Neural control of airway mucus secretion.

48. Kings College Department of Pharmacy Postgraduate Seminars, London. Neuromodulation of airway mucus secretion.

49. Republic of China Society of Pulmonary and Critical Care Medicine, Taipei and Kaohsiung, Taiwan. Airway hypersecretion.

50. The Speywood Laboratory, St George's Hospital Medical School, London. NANC neurones: physiology and pathophysiology.

51. Schering-Plough Research Institute, Kenilworth, New Jersey, U.S.A. Airway mucus hypersecretion: basic mechanisms and potential therapy.

52. The Rayne Institute, University College London. 'Inhibitory neural control of airway secretion'.

53. Department of Pharmacy, University of Brighton. Inhibiting neurogenic airway mucus secretion.

54. Rhône-Poulenc Rorer, Dagenham. Inhibition of airway mucus secretion: new approaches.

1996

55. NHLI Lunchtime Research Overview. 'Inhibition of neurogenic mucus secretion and microanatomy of exocytosis'.

56. Second National Conference on Asthma Education and Management, Kensington New Town Hall, London. How do we define bioequivalence?

57. Peptide Therapeutics, Cambridge. Airway neural actions and the effect of nedocromil.

58. The 9th Combined Meeting of the World Congress for Bronchology and World Congress for Bronchoesophagology, Taipei, Taiwan. Inhibition of airway mucus secretion: novel developments.

59. National Asthma Campaign Lunchtime Seminar, London. Mucus secretion in asthma: what to do about it?

60. Boehringer Ingelheim COPD Meeting, Weisbaden, Germany. Neurogenic inflammation.

61. Department of Pharmacology Research Seminar, King's College, London. Helping to take the sting out of asthma.

62. National Asthma Campaign Grantholders Symposium, NHLI, London. Inhibition of airway neurogenic mucus secretion.

63. New Jersey Thoracic Society and Pulmonary Research Group, Scientific Session: Symposium on 'Pathology and Treatment of COPD,' New Brunswick, USA. Neuromodulation of airway mucous secretions.
Meeting accredited for Continuing Medical Education (CME).

64. Children's Research Institute, Washington DC, USA. Neuromodulation of airway mucin secretion.

65. The Royal Society of Medicine, London, meeting on 'The Role of Mucus in Respiratory Diseases.' *In vivo* preclinical test models for studying mucus.
CME accredited meeting.

1997

66. Roche Bioscience, Palo Alto, USA. Neurogenic airway mucus secretion: mechanisms and modulation.

67. Zambon Italia, Milan, Italy. Airway mucus secretion: mechanisms and modulation.

1998

68. Institut Pasteur, Paris, France. Airway mucus: models of secretion and hypersecretion.

69. Bayer plc, Slough. Neural control of airway mucus secretion: excitatory and inhibitory mechanisms.

70. University of Cardiff, Wales: BALR symposium on 'Respiratory Tract Epithelium and its Secretions.' 'Goblet cells: exocytosis and plasticity'.

71. Department of Medical Oncology, Charing Cross Hospital, London. Neuroregulation of airway mucus secretion.

72. Faculty of Science and Technology, North East Surrey College of Technology (NESCOT). 'Neuroregulation of airways secretion: therapeutic prospects in asthma'.

1999

73. The Guy's, Kings College & St Thomas' Hospitals Medical & Dental School, London. 'Mucin gene expression in airway inflammation'.

74. Novartis Horsham Research Centre, West Sussex. Airway mucus secretion and hypersecretion: basic mechanisms and therapeutic prospects.

75. Madrid, Spain: European Respiratory Society Annual Congress. Neural control of mucus hypersecretion.

76. Lake Garda, Italy: International Meeting on Mucus and Mucociliary Interactions. Differential mucin gene expression in airways of allergic rats.

77. Taipei, Taiwan: 1st International Conference on Immunopharmacology of Bronchial Asthma. Mucus hypersecretion in bronchial asthma.

2000

78. Parke-Davis Institut de Recherche Jouveinal, Paris, France. Mucus secretion in the airways: role and consequences in pathological conditions.

79. Novartis Foundation Symposium No. 234, 'Chronic Obstructive Pulmonary Disease: Pathogenesis to Treatment', London. 'Mucus hypersecretion'.

80. AstraZeneca R&D Charnwood, Leics. 'Neural control and mucus hypersecretion in asthma and COPD'.

81. GlaxoWellcome R&D, Stevenage, Herts. Airway hypersecretion in asthma and COPD: clinical aspects and experimental models.

82. AstraZeneca, Lund, Sweden. Airway mucus: pathophysiology and pharmacotherapy.

83. 'COPD – Pathogenesis to Therapy' (Allen & Hanburys COPD Symposium), Edinburgh. 'Mucus hypersecretion'.

2001

84. Events in Respiratory Medicine, Respiratory Grand Round (NHLI and Royal Brompton & Harefield NHS Trust). 'Mucus hypersecretion: a distinct cholinergic disease?' (with Professor K.F. Chung).

85. Berlin, Germany: The XXth Congress of the European Academy of Allergology and Clinical Immunology. 'Airway mucosa: secretory cells, mucus and mucin genes'.

86. Roche Bioscience, Palo Alto, California, U.S.A. 'Mucus hypersecretion in asthma and COPD: pathophysiology and models'.

87. Centre for Respiratory Research, The Rayne Institute, University College London. 'Mucin gene expression in airway inflammation'.

88. Department of Cell and Molecular Biology, University of Lund, Lund, Sweden. 'Airway mucin gene expression: pathophysiology and models'.

2002

89. National Clinical Coding Conference, London. 'Mucous hypersecretion in asthma and COPD'.

90. Merck Frosst Canada, Quebec, Canada. 'Mucus hypersecretion in asthma and COPD: pathophysiology, models and treatment'.

91. 'COPD 3', International Convention Centre, Birmingham. 'Mucus regulation'.

92. 2002 International Meeting of the Interest Group for Cilia, Mucus, and Mucociliary Interactions and the PCD Foundation. Wyndham Miami Beach Resort, Miami, U.S.A. 'Airway mucus hypersecretion and considerations for rational novel therapy'.

2003

93. AstraZeneca, Charnwood, Loughborough. 'Airway mucus hypersecretion: models and therapies'.

94. The Triangle of Asthma Management: Patient-Substance-Inhaler. 'Airway secretions'. NHLI, Imperial College London.

95. University of Sunderland, Molecular Biology of the Cell Forum Seminar. 'Airway mucus hypersecretion: basic mechanisms and novel pharmacotherapy'.

96. British Society for Allergy and Clinical Immunology, East Midlands Conference Centre, Nottingham. 'Neuronal control of airway mucus secretion'.

97. University of Brighton: BALR symposium on Airway Epithelial Defence Mechanisms. 'Regulation of airway mucin secretion: stimulation and inhibition in experimental models and human disease'.

98. 6th Annual Conference on COPD, Management Forum, London. 'Mucus hypersecretion in COPD: pathophysiology and novel treatments'.

99. Centre for Applied Microbiological Research, Porton Down. 'Airway mucus secretion: stimulation and rational approaches to inhibition of hypersecretion'.

2004

100. Oxagen Limited, Oxford. 'Airway mucus hypersecretion: models and pharmacotherapeutics'.

101. COPD: The Important Questions III. Therapeutic Interventions Now and in the Future, Marbella, Spain. 'Mucoregulators'.

102. The Breathing Club: All Change in COPD. Chantilly, France. 'Mucus regulation and treatment'.

103. Asthma & Allergy Research Group, NHLI, Imperial College London. 'Mucus regulation and treatment in asthma, COPD and allergic rhinitis'.

104. Centre for Respiratory Research, The Rayne Institute, University College London. 'Airway mucus hypersecretion: new pharmacotherapy in asthma and COPD'.

105. Annual Conference on COPD, Management Forum, London. 'Mucus hypersecretion and modifying agents'.

106. Heatherwood and Wexham NHS Trust Medical Academic Half Day, Heatherwood Hospital, Ascot. 'Airway mucus hypersecretion: basic mechanisms and rationales for novel therapy'.

107. British Thoracic Society, Winter Meeting, London. 'Targeting mucus production in lung disease'.

2005

108. Department of Academic Respiratory Medicine, St. Bartholomew's Hospital, London. 'Mucus hypersecretion in COPD'.

109. Brompton Hospital Respiratory Grand Round. 'Inhibiting airway mucus secretion: botulism for the lungs?'

110. Second Shanghai International Symposium on Respiratory Diseases, Zhongshan Hospital, Fudan University, Shanghai, China. 'Airway mucus hypersecretion: rationales for pharmacotherapy'.

111. University of Leicester. 'Botulism for the lungs: inhibiting airway mucus secretion'.

Attorney Docket No. 5051-451IP PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re: Li et al.
Serial No.: 09/914,020
Filed: December 31, 2001
For: *Methods and Compositions for Altering Mucus Secretion*

Confirmation No.: 8515
Art Unit: 1633
Examiner: J. Epps-Ford

Date: June 7, 2006

Mailstop Amendment
Commissioner for Patents
PO Box 1450
Alexandria, VA 22313-1450

ATTACHMENT C

Exhibit 2

"Global Initiative for Asthma: Pocket Guide for Asthma Management and Prevention"

POCKET GUIDE FOR ASTHMA MANAGEMENT AND PREVENTION

A Pocket Guide for Physicians and Nurses

Updated 2005

BASED ON THE WORKSHOP REPORT:
GLOBAL STRATEGY FOR ASTHMA MANAGEMENT AND PREVENTION
(UPDATED 2005)

GLOBAL INITIATIVE FOR ASTHMA

GLOBAL INITIATIVE FOR ASTHMA

Executive Committee (2005)

Paul O'Byrne, M.D., Canada, *Chair*
Eric D. Bateman, M.D., South Africa
William Busse, M.D., U.S.A.
Jean Bousquet, M.D., Ph.D., France
Tim Clark, M.D., U.K.
Pierluigi Paggario, Italy
Ken Ohta, M.D., Japan
Soren Pedersen, M.D., Denmark
Manuel Soto-Quiroz, Costa Rica
Raj Singh, M.D., India
Wan Cheng Tan, M.D.,Canada

GINA Assembly (2005)

Wan Cheng Tan, M.D., Canada, *Chair*

GINA Assembly members from 45 countries (names are listed on the GINA website: www.ginasthma.org

Updated from the NHLBI/WHO Workshop Report:
Global Strategy for Asthma Management and Prevention **issued January, 1995, and revised 2002.**
NIH Publication No. 02-3659
GINA reports are available on http://www.ginasthma.org

TABLE OF CONTENTS

PREFACE

Asthma is a major cause of chronic morbidity and mortality throughout the world. The **Global Initiative for Asthma** was created to increase awareness of asthma among health professionals, public health authorities, and the general public, and to improve prevention and management through a concerted worldwide effort. The Initiative prepares scientific reports on asthma, encourages dissemination and adoption of the reports, and promotes international collaboration on asthma research.

While asthma has been recognized for many years, public health officials are concerned about recent and continuing increases in its prevalence. The **Global Initiative for Asthma** offers a framework for asthma management that can be adapted to local health care systems and resources. Educational tools, such as laminated cards, or computer-based learning programs can be prepared that are tailored to these systems and resources.

The **Global Initiative for Asthma** program publications include:

- *Workshop Report: Global Strategy for Asthma Management and Prevention* (updated 2005). Scientific information and recommendations for asthma programs.
- *Pocket Guide for Asthma Management and Prevention.* Summary of patient care information for primary health care professionals. (updated 2005).
- *Pocket Guide for Asthma Management and Prevention in Children.* Summary of patient care information for pediatricians and other health care professionals. (updated 2005).
- *What You and Your Family Can Do About Asthma.* An information booklet for patients and their families.

Publications are available from http://www.ginasthma.org

This *Pocket Guide* has been developed from the *Workshop Report: Global Strategy for Asthma Management and Prevention* (updated 2005). Technical discussions of asthma, evidence levels, and specific citations from the scientific literature are included in the Workshop Report.

Acknowledgements:

Grateful acknowledgement is given for unrestricted educational grants from Altana, Andi-Ventis, AstraZeneca, Aventis, Bayer, Boehringer Ingelheim, Chiesi Group, GlaxoSmithKline, Merck, Sharp & Dohme, Mitsubishi-Pharma Corporation, Nikken Chemicals Co., LTD., Novartis, Schering-Plough International and Viatris. The generous contributions of these companies assured that the workshop participants could meet together and publications could be printed for wide distribution. However, the workshop participants are solely responsible for the statements and conclusions in the publications.

Attorney Docket No. 5051-451IP PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Inventor: Li et al. Confirmation No.: 8515
Serial No.: 09/914,020 Art Unit: 1633
Filed: December 31, 2001 Examiner: J. Epps-Ford
For: *Methods and Compositions for Altering Mucus Secretion*

Date: June 7, 2006

Mailstop Amendment
Commissioner for Patents
PO Box 1450
Alexandria, VA 22313-1450

ATTACHMENT C

Exhibit 3

"National Institute for Clinical Excellence (NICE): Chronic Obstructive Pulmonary Disease, Management of Chronic Obstructive Pulmonary Disease in Adults in Primary and Secondary care"

National Institute for Clinical Excellence

Chronic obstructive pulmonary disease

Management of chronic obstructive pulmonary disease in adults in primary and secondary care

Clinical Guideline 12

February 2004

Developed by the National Collaborating Centre for

Clinical Guideline 12
Chronic obstructive pulmonary disease
Management of chronic obstructive pulmonary disease in adults in primary and secondary care

Issue date: February 2004

This document, which contains the Institute's full guidance on the management of chronic obstructive pulmonary disease in adults, is available from the NICE website (www.nice.org.uk/CG012NICEguideline).

An abridged version of this guidance (a 'quick reference guide') is also available from the NICE website (www.nice.org.uk/CG012quickrefguide). Printed copies of the quick reference guide can be obtained from the NHS Response Line: telephone 0870 1555 455 and quote reference number N0462.

Information for the Public is available from the NICE website or from the NHS Response Line (quote reference number N0463 for a version in English and N0464 for a version in English and Welsh).

The quick reference guide for this guideline has been distributed to the following:

- PCT chief executives
- NHS trust chief executives in England and Wales
- Clinical governance leads in England and Wales
- Audit leads in England and Wales
- Local health board chief executives
- Medical and nursing directors in England and Wales
- NHS trust, PCT and LHB libraries in England and Wales
- Consultants in occupational health medicine in England and Wales
- Consultants in rehabilitation medicine in England and Wales
- Consultants in respiratory medicine in England and Wales
- Consultants in elderly care in England and Wales
- Clinical directors for physiotherapy in England and Wales
- Directorate nurse managers for occupational therapy in England and Wales
- Directorate nurse managers for rehabilitation in England and Wales
- Directorate nurse managers for respiratory medicine in England and Wales
- Respiratory nurse specialists in England and Wales
- Senior pharmacists and pharmaceutical advisors in England and Wales
- GPs in England and Wales
- Senior health visitors, practice nurses and community nurses in England and Wales
- Strategic health authority chief executives in England and Wales
- Directors of directorates of health and social care
- NHS Director Wales
- Chief Executive of the NHS in England
- NHS Executive regional directors
- Patient advocacy groups
- Commission for Health Improvement
- NHS Clinical Governance Support Team
- Chief Medical, Nursing and Pharmaceutical Officers in England and Wales
- Medical Director & Head of NHS Quality - Welsh Assembly Government
- Representative bodies for health services, professional organisations and statutory bodies and the Royal Colleges

---

This guidance is written in the following context:
This guidance represents the view of the Institute, which was arrived at after careful consideration of the evidence available. Health professionals are expected to take it fully into account when exercising their clinical judgement. The guidance does not, however, override the individual responsibility of health professionals to make decisions appropriate to the circumstances of the individual patient, in consultation with the patient and/or guardian or carer.

National Institute for Clinical Excellence

MidCity Place
71 High Holborn
London WC1V 6NA

www.nice.org.uk

ISBN: 1-84257-542-2
Published by the National Institute for Clinical Excellence
February 2004

Contents

Table 4 Assessment of severity of airflow obstruction according to FEV_1 as a percentage of the predicted value

Severity	FEV_1
Mild airflow obstruction	50–80% predicted
Moderate airflow obstruction	30–49% predicted
Severe airflow obstruction	< 30% predicted

1.1.6 Identification of early disease

1.1.6.1 Spirometry should be performed in patients who are over 35, current or ex-smokers, and have a chronic cough. D

1.1.6.2 Spirometry should be considered in patients with chronic bronchitis. A significant proportion of these will go on to develop airflow limitation. B

1.1.7 Referral for specialist advice

1.1.7.1 It is recommended that referrals for specialist advice are made when clinically indicated. Referral may be appropriate at all stages of the disease and not solely in the most severely disabled patients (see Table 5). D

1.1.7.2 Patients who are referred do not always have to be seen by a respiratory physician. In some cases they may be seen by members of the COPD team who have appropriate training and expertise. D

1.2 Managing stable COPD

1.2.1 Smoking cessation

1.2.1.1 An up-to-date smoking history, including pack years smoked (number of cigarettes smoked per day, divided by 20, multiplied by the number of years smoked), should be documented for everyone with COPD. D

1.2.1.2 All COPD patents still smoking, regardless of age, should be encouraged to stop, and offered help to do so, at every opportunity. A

1.2.1.3 Unless contraindicated, bupropion or nicotine replacement therapy combined with an appropriate support programme should be used to optimise smoking quit rates for people with COPD. B

be referred for consideration of lung volume reduction surgery if they meet all of the following criteria:

- FEV_1 more than 20% predicted
- $PaCO_2$ less than 7.3 kPa
- upper lobe predominant emphysema
- T_LCO more than 20% predicted.

1.2.12.3 Patients with severe COPD who remain breathless with marked restrictions of their activities of daily living despite maximal medical therapy should be considered for referral for assessment for lung transplantation, bearing in mind comorbidities and local surgical protocols. Considerations include: C

- age
- FEV_1
- $PaCO_2$
- homogeneously distributed emphysema on CT scan
- elevated pulmonary artery pressures with progressive deterioration.

1.2.13 Alpha-1 antitrypsin replacement therapy

1.2.13.1 Alpha-1 antitrypsin replacement therapy is not recommended in the management of patients with alpha-1 antitrypsin deficiency (see also recommendation 1.1.3.3). D

1.2.14 Mucolytic therapy

1.2.14.1 Mucolytic drug therapy should be considered in patients with a chronic cough productive of sputum. B

1.2.14.2 Mucolytic therapy should be continued if there is symptomatic improvement (for example, reduction in frequency of cough and sputum production). D

1.2.15 Anti-oxidant therapy

1.2.15.1 Treatment with alpha-tocopherol and beta-carotene supplements, alone or in combination, is not recommended. A

1.2.16 Anti-tussive therapy

1.2.16.1 Anti-tussive therapy should not be used in the management of stable COPD. D

Attorney Docket No. 5051-451IP PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re: Li et al. Confirmation No.: 8515
Serial No.: 09/914,020 Art Unit: 1633
Filed: December 31, 2001 Examiner: J. Epps-Ford
For: *Methods and Compositions for Altering Mucus Secretion*

Date: June 7, 2006

Mailstop Amendment
Commissioner for Patents
PO Box 1450
Alexandria, VA 22313-1450

ATTACHMENT C

Exhibit 4

Global Initiative for Chronic Obstructive Lung Disease, Global Strategy for the Diagnosis, Management, and Prevention of Chronic Obstructive Pulmonary Disease

GLOBAL INITIATIVE FOR CHRONIC OBSTRUCTIVE LUNG DISEASE

GLOBAL STRATEGY FOR THE DIAGNOSIS, MANAGEMENT, AND PREVENTION OF CHRONIC OBSTRUCTIVE PULMONARY DISEASE

NHLBI/WHO WORKSHOP REPORT

(Based on an April 1998 meeting)

NATIONAL INSTITUTES OF HEALTH
National Heart, Lung, and Blood Institute
Publication Number 2701
April 2001

TABLE OF CONTENTS

Figure 4-5. Characteristics of Inflammation in COPD and Asthma		
	COPD	**Asthma**
Cells	• Neutrophils • Large increase in macrophages • Increase in $CD8^+$ T lymphocytes	• Eosinophils • Small increase in macrophages • Increase in $CD4^+$ Th2 lymphocytes • Activation of mast cells
Mediators	• LTB4 • IL-8 • TNF-α	• LTD4 • IL-4, IL-5 • (Plus many others)
Consequences	• Squamous metaplasia of epithelium • Parenchymal destruction • Mucus metaplasia • Glandular enlargement	• Fragile epithelium • Thickening of basement membrane • Mucus metaplasia • Glandular enlargement
Response to Treatment	• Glucocorticosteroids have little or no effect	• Glucocorticosteroids inhibit inflammation

Inflammation and COPD Risk Factors

The connection between cigarette smoke and inflammation has been most extensively studied[41-52]. Cigarette smoke activates macrophages and epithelial cells to produce TNF-α and may also cause macrophages to release other inflammatory mediators, including IL-8 and LTB4[53,54].

Inflammation is present in the lungs of smokers without a diagnosis of COPD. This inflammation is similar to, but less intense than, the inflammation in the lungs of patients with COPD. For example, induced sputum studies show that smokers without COPD have a greater proportion of neutrophils in their lungs than age-matched nonsmokers, but a smaller proportion than COPD patients[4,9]. Thus, the inflammation characteristic of COPD is thought to represent an exaggeration of a normal, protective response to inhalational exposures.

However, not all smokers develop COPD, and why the normal, protective inflammatory response becomes an exaggerated, harmful one in some smokers is poorly understood. Presumably the inflammation caused by cigarette smoking interacts with other host or environmental factors to produce the excess decline in lung function that results in COPD[55]. Inflammatory changes are also present in bronchial biopsies in ex-smokers, suggesting that the inflammatory response in COPD may persist even in the absence of continuous exposure to risk factors[56].

A number of studies have demonstrated that a variety of particulates (e.g., diesel exhaust, grain dust) can initiate respiratory tract inflammation[57-61]. It is likely that indoor air pollution derived from the burning of biomass fuels will prove to have similar effects.

Proteinase-Antiproteinase Imbalance

Laurell and Eriksson observed in 1963 that individuals with a hereditary deficiency of the serum protein alpha-1 antitrypsin, which inhibits a number of serine proteinases such as neutrophil elastase, are at increased risk of developing emphysema[62]. Elastin, the target of neutrophil elastase, is a major component of alveolar walls, and elastin fragments may perpetuate inflammation by acting as potent chemotactic agents for macrophages and neutrophils. These observations led to the hypothesis that an imbalance between proteinases and endogenous antiproteinases results in lung destruction.

Based on many observations, it now seems clear that an imbalance of proteinases and antiproteinases may involve either increased production or activity of proteinases, or inactivation or reduced production of antiproteinases. Often, the imbalance is a consequence of the inflammation induced by inhalational exposures. For example, macrophages, neutrophils, and airway epithelial cells release a combination of proteinases. The imbalance may also be caused by a decrease of antiproteinase activity by oxidative stress (itself a consequence

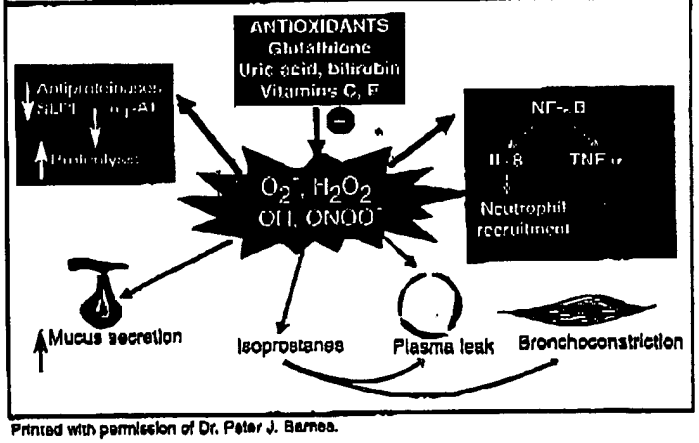
of inflammation), cigarette smoke[63,64], and possibly other COPD risk factors.

The concept has also been expanded to include additional proteinases and antiproteinases. While neutrophil elastase is likely to be the major proteinase involved in lung destruction in alpha-1 antitrypsin deficiency, it may not be involved in COPD caused by inhalational exposures. Additional proteinases that have been implicated in COPD include neutrophil cathepsin G, neutrophil proteinase-3, cathepsins released from macrophages (specifically cathepsins B, L, and S), and various matrix metalloproteinases (MMPs)[65]. These proteinases are capable of degrading elastin and also collagen, another main component of alveolar walls. Some proteinases, such as neutrophil elastase[66] and neutrophil proteinase-3[67], induce mucus secretion, and neutrophil elastase also produces mucus gland hyperplasia[68]. Thus, proteinases may be involved in mucus hypersecretion as well as parenchymal destruction. Antiproteinases thought to be involved in COPD include, in addition to alpha-1 antitrypsin, secretory leukoproteinase inhibitor (SLPI) and tissue inhibitors of MMPs (TIMPs).

Oxidative Stress

There is increasing evidence that an oxidant/antioxidant imbalance, in favor of oxidants, occurs in COPD. (The process is summarized in **Figure 4-6.**) Markers of oxidative stress have been found in the epithelial lining fluid, breath, and urine of cigarette smokers and patients with COPD. For example, hydrogen peroxide (H_2O_2) and nitric oxide (NO) are direct measures of oxidants generated by cigarette smoking or released from inflammatory leukocytes and epithelial cells. H_2O_2 is increased in the breath of patients with stable COPD and during acute exacerbations[69], and NO is increased in the breath during exacerbations of COPD[70]. A prostaglandin

Figure 4-6. Increased Oxidative Stress in COPD



Printed with permission of Dr. Peter J. Barnes.

isomer, isoprostane $F_2\alpha$-III, which is formed by free radical peroxidation of arachidonic acid and believed to be an *in vivo* biomarker of lung oxidative stress, is increased in both breath condensates[71] and urine[72] in COPD patients compared to healthy controls and is increased even more during exacerbations.

Oxidative stress contributes to COPD in a variety of ways. Oxidants can react with, and damage, a variety of biological molecules, including proteins, lipids, and nucleic acids, and this can lead to cell dysfunction or death, as well as damage to the lung extracellular matrix. In addition to directly damaging the lung, oxidative stress contributes to the proteinase-antiproteinase imbalance both by inactivating antiproteinases (such as alpha-1 antitrypsin and SLPI) and by activating proteinases (such as MMPs). Oxidants also promote inflammation, for example by activating the transcription factor NF-κB, which orchestrates the expression of multiple inflammatory genes thought to be important in COPD such as IL-8 and TNF-α. Finally, oxidative stress may contribute to reversible airway narrowing. H_2O_2 constricts airway smooth muscle *in vitro* and isoprostane $F_2\alpha$-III is a potent constrictor of human airways[73].

PATHOLOGY

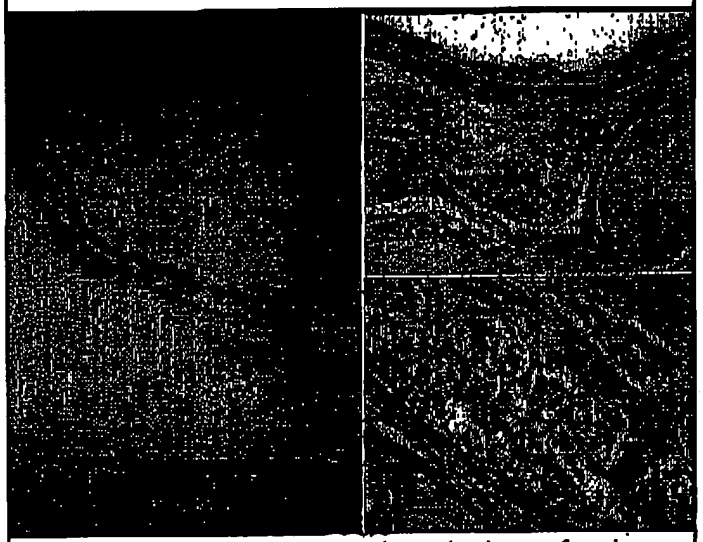
Pathological changes characteristic of COPD are found in the central airways, peripheral airways, lung parenchyma, and pulmonary vasculature[74]. The various lesions are a result of chronic inflammation in the lung, which in turn is initiated by the inhalation of noxious particles and gases such as those present in cigarette smoke. The lung has natural defense mechanisms and a considerable capacity to repair itself, but the working of these mechanisms may be affected by genetic traits (e.g., alpha-1 antitrypsin deficiency) or exposure to other environmental risk factors (e.g., infection, atmospheric pollution)[75], as well as by the chronic nature of the inflammation and repeated nature of the injury.

Central Airways

The central airways include the trachea, bronchi, and bronchioles greater than 2-4 mm in internal diameter. In patients with chronic bronchitis, an inflammatory exudate of fluid and cells infiltrates the epithelium lining the central airways and associated glands and ducts[2,42]. The predominant cells in this inflammatory exudate are macrophages and $CD8^+$ T lymphocytes[2,76]. Chronic inflammation in the central airways is also associated with an increase in the number (metaplasia) of epithelial goblet and squamous cells; dysfunction, damage, and/or loss of cilia; enlarged submucosal mucus-secreting

glands[77]; an increase in the amount of smooth muscle and connective tissue in the airway wall[78]; degeneration of the airway cartilage[79,80]; and mucus hypersecretion. The mechanisms of mucus gland hypertrophy and goblet cell metaplasia have not yet been identified, but animal studies[81,82] show that irritants including cigarette smoke[83] can produce these changes. The various pathological changes in the central airways (**Figure 4-7**) are responsible for the symptoms of chronic cough and sputum production, which identify people at risk for COPD and may continue to be present throughout the course of the disease. Thus, these pathological changes may be present either on their own or in combination with the changes in the peripheral airways and lung parenchyma described below.

Figure 4-7. Pathological Changes of the Central Airways in COPD



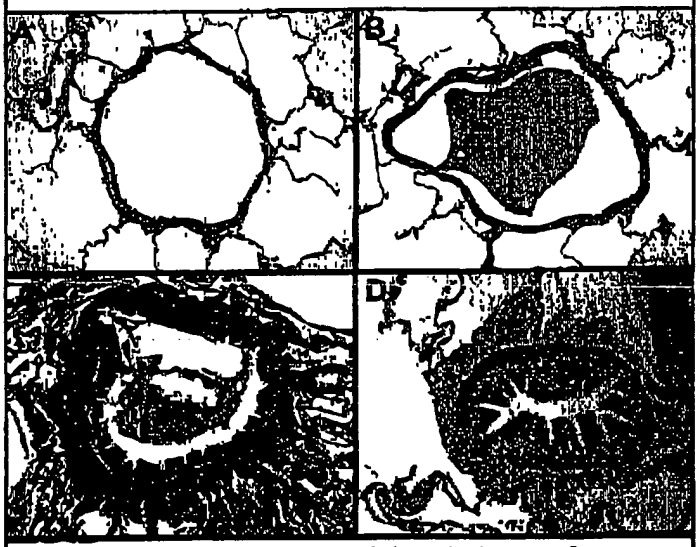
(A) shows a central bronchus from the lung of a cigarette smoker with normal lung function. Only small amounts of muscle are present and the epithelial glands are small. This contrasts sharply with a diseased bronchus (B), where the muscle appears as a thick bundle and the glands are enlarged. (C) shows these enlarged glands at a higher magnification. There is evidence of a chronic inflammatory process involving polymorphonuclear (arrowhead) and mononuclear cells, including plasma cells (arrow).

Printed with permission of Dr. James C. Hogg and Stuart Greene.

Peripheral Airways

The peripheral airways include small bronchi and bronchioles that have an internal diameter of less than 2 mm (**Figure 4-8**). The early decline in lung function in COPD is correlated with inflammatory changes in the

Figure 4-8. Pathological Changes of the Peripheral Airways in COPD



Histological sections of peripheral airways from patients who are cigarette smokers. (A) is a nearly normal airway; (B) shows a plug of mucoid exudate in the lumen with little or no evidence of inflammation in the wall; (C) shows the presence of an inflammatory exudate in the wall and lumen of the airway; and (D) shows an airway with reduced lumen, structual reorganization of the airway wall, increased smooth muscle, and depostition of peribronchial connective tissue.

Printed with permission of Dr. James C. Hogg and Stuart Greene.

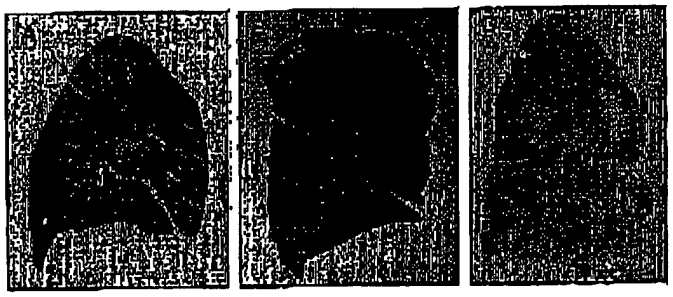
peripheral airways, similar to those that occur in the central airways: exudate of fluid and cells in the airway wall and lumen, goblet and squamous cell metaplasia of the epithelium[43], edema of the airway mucosa due to inflammation, and excess mucus in the airways due to goblet cell metaplasia.

However, the most characteristic change in the peripheral airways of patients with COPD is airway narrowing. Inflammation initiated by cigarette smoking[45] and other risk factors[75] leads to repeated cycles of injury and repair of the walls of the peripheral airways. Injury is caused either directly by inhaled toxic particles and gases such as those found in cigarette smoke, or indirectly by the action of inflammatory mediators; this injury then initiates repair processes. Although airway repair is only partly understood, it seems likely that disordered repair processes can lead to tissue remodeling with altered structure and function. Cigarette smoke may impair lung repair mechanisms, thereby further contributing to altered lung structure[84-86]. Even normal lung repair mechanisms

can lead to airway remodeling because tissue repair in the airways, as elsewhere in the body, may involve scar tissue formation. In any case, this injury-and-repair process results in a structural remodeling of the airway wall, with increasing collagen content and scar tissue formation, that narrows the lumen and produces fixed airways obstruction[87]. The peripheral airways become the major site of airways obstruction in COPD, and direct measurements of peripheral airways resistance[88] show that the structural changes in the airway wall are the most important cause of the increase in peripheral airways resistance in COPD. Inflammatory changes such as airway edema and mucus hypersecretion also contribute to airway narrowing in COPD. So does loss of elastic recoil, but fibrosis of the small airways plays the largest role.

Fibrosis in the peripheral airways, as elsewhere in the body, is characterized by the accumulation of mesenchymal cells (fibroblasts and myofibroblasts) and extracellular connective tissue matrix. Several cell types including mononuclear phagocytes and epithelial cells may produce mediators that drive this process. The mediators that drive the accumulation of these cells and of the matrix are incompletely defined, but it is likely that several mediators including TGF-β, ET-1, insulin-like growth factor-1, fibronectin, platelet-derived growth factor (PDGF), and others are involved[89].

Figure 4-9. Normal and Emphysematous Lungs



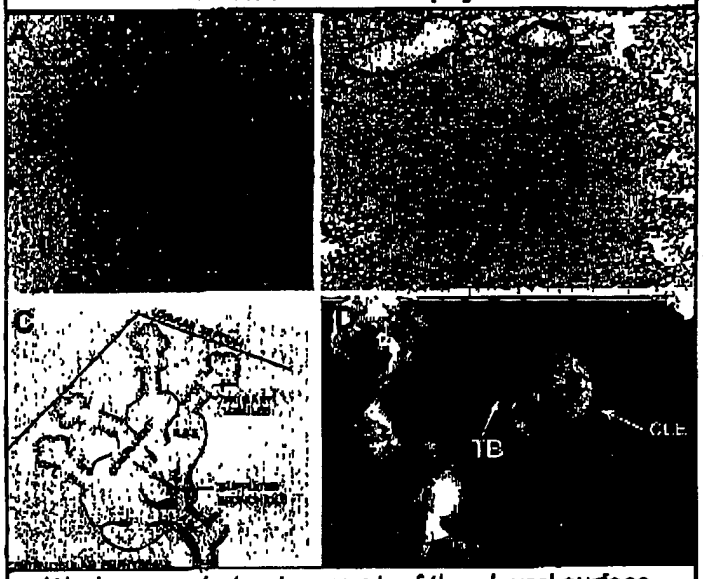
Photomicrographs of paper-mounted whole lung sections prepared from (A) a normal lung, (B) a lung with mild centrilobular emphysema, and (C) a lung with severe panacinar emphysema. Note that the centrilobular form affects mainly the upper lung regions whereas the panacinar form is more apparent in the lower lung regions.

Printed with permission of Dr. James C. Hogg and Stuart Greene.

Lung Parenchyma

The lung parenchyma includes the gas exchanging surface of the lung (respiratory bronchioles and alveoli) and the pulmonary capillary system (**Figure 4-9**). The most

Figure 4-10. Normal Respiratory Bronchioles and Centrilobular Emphysema



(A) shows a photomicrograph of the pleural surface of a normal lung, with a secondary lobule defined by a connective tissue septum (solid arrow) and several terminal bronchioles (TB) filled with opaque material. (B) shows a low-power photomicograph of a normal terminal bronchiole (TB) branching into a respiratory bronchiole (RB), which eventually end in alveolar ducts (AD). (C) is a schematic diagram of centrilobular emphysema and (D) shows the bronchographic appearance of this lesion (TB=terminal bronchiole; CLE=centrilobular emphysema).

Printed with permission of Dr. James C. Hogg and Stuart Greene.

common type of parenchymal destruction in COPD patients is the centrilobular form of emphysema (**Figure 4-10**), which involves dilatation and destruction of the respiratory bronchioles[90]. These lesions occur more frequently in the upper lung regions in milder cases, but in advanced disease they may appear diffusely throughout the entire lung and also involve destruction of the pulmonary capillary bed. Panacinar emphysema, which extends throughout the acinus, is the characteristic lesion seen in alpha-1 antitrypsin deficiency and involves dilatation and destruction of the alveolar ducts and sacs as well as the respiratory bronchioles. It tends to affect the lower more than upper lung regions. Because this process usually affects all of the acini in the secondary lobule, it is also referred to as panlobular emphysema. The primary mechanism of lung parenchyma destruction, in both smoking-related COPD and alpha-1 antitrypsin deficiency, is thought to be an imbalance of endogenous proteinases and antiproteinases in the lung. Oxidative stress, another consequence of inflammation, may also contribute[91].

Figure 4-11. Pathological Changes of the Pulmonary Vasculature in COPD

Photomicrographs of small (A) and large (B) vessels in the lung of a heavy smoker with normal lung function, and small (C) and large (D) vessels in the lung of a patient with severe emphysema. Note that the smaller vessel has thicker walls (compare arrows in A and C) and that the larger vessel has a thicker media (compare arrows in B and D) in the patient with severe emphysema. (L=vessel lumen; magnification bars=100μ).

Printed with permission of Dr. James C. Hogg and Stuart Greene.

Pulmonary Vasculature

Pulmonary vascular changes in COPD (**Figure 4-11**) are characterized by a thickening of the vessel wall that begins early in the natural history of the disease, when lung function is reasonably well maintained and pulmonary vascular pressures are normal at rest[92]. Endothelial dysfunction of the pulmonary arteries, which may be caused directly by cigarette smoke products[93] or indirectly by inflammatory mediators[14], occurs early in COPD[94]. Since endothelium plays an important role in regulating vascular tone and cell proliferation, it is likely that endothelial dysfunction might initiate the sequence of events that results ultimately in structural changes. Thickening of the intima is the first structural change[92], followed by an increase in vascular smooth muscle and the infiltration of the vessel wall by inflammatory cells, including macrophages and $CD8^+$ T lymphocytes[14]. These structural changes are correlated with an increase in pulmonary vascular pressure that develops first with exercise and then at rest. As COPD worsens, greater amounts of smooth muscle, proteoglycans, and collagen[93] further thicken the vessel wall. In advanced disease, the changes in the muscular arteries may be associated with emphysematous destruction of the pulmonary capillary bed.

PATHOPHYSIOLOGY

Pathological changes in COPD lead to corresponding physiological abnormalities that usually become evident first on exercise and later also at rest. Physiological changes characteristic of the disease include mucus hypersecretion, ciliary dysfunction, airflow limitation, pulmonary hyperinflation, gas exchange abnormalities, pulmonary hypertension, and cor pulmonale, and they usually develop in this order over the course of the disease. In turn, various physiological abnormalities contribute to the characteristic symptoms of COPD — chronic cough and sputum production and dyspnea.

Mucus Hypersecretion and Ciliary Dysfunction

Mucus hypersecretion in COPD is caused by the stimulation of the enlarged mucus secreting glands and increased number of goblet cells by inflammatory mediators such as leukotrienes, proteinases, and neuropeptides. Ciliated epithelial cells undergo squamous metaplasia leading to impairment in mucociliary clearance mechanisms. These changes are usually the first physiological abnormalities to develop in COPD, and can be present for many years before any other physiological abnormalities develop.

Airflow Limitation and Pulmonary Hyperinflation

Expiratory airflow limitation is the hallmark physiological change of COPD. The airflow limitation characteristic of COPD is primarily irreversible, with a small reversible component. Several pathological characteristics contribute to airflow limitation and changes in pulmonary mechanics, as summarized in **Figure 4-12**. The irreversible component of airflow limitation is primarily due to remodeling[42,43,87,88,96,97] — fibrosis and narrowing — of the small airways that produces fixed airways obstruction and a consequent increase in airways resistance. The sites of airflow limitation in COPD are the smaller conducting airways, including bronchi and bronchioles less than 2 mm in internal diameter. In the normal lung, resistance of these smaller airways makes up a small

COMPONENT 3: MANAGE STABLE COPD

KEY POINTS:

- The overall approach to managing stable COPD should be characterized by a stepwise increase in treatment, depending on the severity of the disease.
- For patients with COPD, health education can play a role in improving skills, ability to cope with illness, and health status. It is effective in accomplishing certain goals, including smoking cessation (**Evidence A**).
- None of the existing medications for COPD has been shown to modify the long-term decline in lung function that is the hallmark of this disease (**Evidence A**). Therefore, pharmacotherapy for COPD is used to decrease symptoms and/or complications.
- Bronchodilator medications are central to the symptomatic management of COPD (**Evidence A**). They are given on an as-needed basis or on a regular basis to prevent or reduce symptoms.
- The principal bronchodilator treatments are β_2-agonists, anticholinergics, theophylline, and a combination of these drugs (**Evidence A**).
- Regular treatment with inhaled glucocorticosteroids should only be prescribed for symptomatic COPD patients with a documented spirometric response to glucocorticosteroids or in those with an FEV_1 < 50% predicted and repeated exacerbations requiring treatment with antibiotics or oral glucocorticosteroids (**Evidence B**).
- Chronic treatment with systemic glucocorticosteroids should be avoided because of an unfavorable benefit-to-risk ratio (**Evidence A**).
- All COPD patients benefit from exercise training programs, improving with respect to both exercise tolerance and symptoms of dyspnea and fatigue (**Evidence A**).
- The long-term administration of oxygen (> 15 hours per day) to patients with chronic respiratory failure has been shown to increase survival (**Evidence A**).

INTRODUCTION

The overall approach to managing stable COPD should be characterized by a stepwise increase in treatment, depending on the severity of the disease. The step-down approach used in the chronic treatment of asthma is not applicable to COPD since COPD is usually stable and very often progressive. Management of COPD involves several objectives (see *Chapter 5, Introduction*) that should be met with minimal side effects from treatment. It is based on an individualized assessment of disease severity (**Figure 5-3-1**) and response to various therapies.

Figure 5-3-1. Factors Affecting the Severity of COPD

- Severity of symptoms
- Severity of airflow limitation
- Frequency and severity of exacerbations
- Presence of one or more complications
- Presence of respiratory failure
- Presence of comorbid conditions
- General health status
- Number of medications needed to manage the disease

The classification of severity (**Figure 1-2**) of stable COPD incorporates an individualized assessment of disease severity and therapeutic response into the management strategy. This classification is a guide that should help health care workers make decisions about the management of COPD in individual patients. Treatment depends on the patient's educational level and willingness to apply the recommended management, on cultural and local conditions, and on the availability of medications.

EDUCATION

Although patient education is generally regarded as an essential component of care for any chronic disease, the role of education in COPD has been poorly studied. Assessment of the value of education in COPD may be difficult because of the relatively long time required to achieve improvements in objective measurements of lung function.

Studies that have been done indicate that patient education alone does not improve exercise performance or lung function[1-4] (**Evidence B**), but it can play a role in improving skills, ability to cope with illness, and health

Attorney Docket No. 5051-451IP PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re: Li et al. Confirmation No.: 8515
Serial No.: 09/914,020 Art Unit: 1633
Filed: December 31, 2001 Examiner: J. Epps-Ford
For: *Methods and Compositions for Altering Mucus Secretion*

Date: June 7, 2006

Mailstop Amendment
Commissioner for Patents
PO Box 1450
Alexandria, VA 22313-1450

ATTACHMENT C

Exhibit 5

Rogers (2004) "Airway mucus hypersecretion in asthma: an undervalued pathology?" Current Opinion in Pharmacology, 4:241-250.

ELSEVIER

Airway mucus hypersecretion in asthma: an undervalued pathology?

Duncan F Rogers

Airway mucus hypersecretion is a feature of many patients with asthma. It is indicative of poor asthma control and contributes to morbidity and mortality. Excess mucus not only obstructs airways but also contributes to airway hyperresponsiveness. Furthermore, asthma might have a specific mucus hypersecretory phenotype. Goblet cell hyperplasia and submucosal gland hypertrophy are shared with other hypersecretory diseases, such as chronic obstructive pulmonary disease; however, some features are different, including mucus plugging, mucus 'tethering' to goblet cells, plasma exudation, and increased amounts of a low charge glycoform of mucin (MUC)5B and the presence of MUC2 in secretions. Experimentally, most of the inflammatory mediators and neural mechanisms implicated in the pathophysiology of asthma impact upon the mucus hypersecretory phenotype. There is currently huge research interest in identifying targets involved in inducing mucus abnormalities, which should lead to the rational design of anti-hypersecretory drugs for treatment of airway mucus hypersecretion in asthma.

Addresses
Thoracic Medicine, National Heart & Lung Institute, Imperial College London, Dovehouse Street, London SW3 6LY, UK
e-mail: duncan.rogers@imperial.ac.uk

Current Opinion in Pharmacology 2004, 4:241–250

This review comes from a themed issue on
Respiratory pharmacology
Edited by Roy Goldie and Peter Henry

1471-4892/$ – see front matter

DOI 10.1016/j.coph.2004.01.011

Abbreviations

COPD	chronic obstructive pulmonary disease
IL	interleukin
MARCKS	myristoylated alanine-rich C kinase substrate

Introduction

Airway mucus hypersecretion is an undervalued pathology. It has long languished as the 'ugly sister' to bronchoconstriction and eosinophilic inflammation in research into the pathophysiology of asthma. However, epidemiological studies demonstrate that mucus is a far from innocent disorder [1]. Indeed, current guidelines on asthma management highlight mucus plugging (Figure 1) alongside bronchoconstriction and inflammation as a cause of airway obstruction and airflow limitation [2]. Consequently, it is important to understand the pathophysiology of mucus hypersecretion in asthma. This should allow identification of therapeutic targets and subsequent rational development of pharmacotherapeutic drugs. This review focuses on the pathophysiology of mucus hypersecretion in asthma by, firstly, describing the mucus hypersecretory phenotype as it pertains to asthma; secondly, assessing the pathophysiological consequences and clinical impact of mucus hypersecretion in asthma; and thirdly, considering the epidemiology of mucus hypersecretion in asthma. The article finishes by outlining conventional and novel therapies for this condition.

Airway mucus

In healthy individuals, a film of slimy liquid protects the airway surface from inhaled 'insult' [3•]. The liquid is referred to as 'mucus' and is a complex non-homogeneous dilute (1–2%) aqueous solution of electrolytes, endogenous and exogenous proteins, lipids and carbohydrates. Mucus forms an upper gel layer and a lower sol layer. Inhaled particles are trapped in the gel and, by transportation on the tips of beating cilia, are removed from the airways: a process termed mucociliary clearance. Mucus contains ~2% mucins [4•], which are high molecular weight glycoproteins that confer the viscoelasticity required for efficient mucus–cilia interaction. Airway mucins are secreted by goblet cells in the surface epithelium [5] and mucous cells in the submucosal glands [6]. Mature mucins are long thread-like molecules composed of monomers joined end to end by disulphide bridges. The mucin monomers comprise a highly glycosylated linear peptide sequence, termed apomucin, which is encoded by specific mucin genes (MUCs). Of the 18 human MUC genes reported to date, MUC5AC and MUC5B gene products are the major gel-forming mucins in airway secretions [4•], although MUC2 might be upregulated in asthma (see below) (Figure 2).

Mucus hypersecretory phenotype in asthma

Airway mucus hypersecretion in asthma has characteristic pathophysiological features. Many of these features, such as sputum production and goblet cell hyperplasia, are common to other hypersecretory respiratory diseases; for example, chronic obstructive pulmonary disease (COPD) and cystic fibrosis. Other features appear to be specifically associated with asthma (see below). Differences in mucus pathophysiology between asthma and COPD have been discussed previously [7], and are summarized in Figure 2. Presumably, differences between the pulmonary inflammatory 'profiles' of asthma and COPD (the former essentially a Th2 lymphocyte-driven eosinophilia, the latter a macrophage-driven neutrophilia) [8] underlie the

Figure 1

Airway mucus plugging in asthma. **(a)** Gross pathology of the lung from an asthmatic patient showing gelatinous plugs blocking (Pb) or partially occluding (Pp) large airways. Courtesy of Dr Catherine Corbishley whose gloved thumb (T) and finger (F) are holding the specimen. **(b)** Histology of an intrapulmonary airway of an asthmatic patient showing occlusion of the lumen by a mucus plug (P) with a marked infiltration of inflammatory cells (arrow). E, epithelium (damaged); G, submucosal gland; M, reticular basement membrane (thickened); V, blood vessel (with evidence of vasodilatation).

variations in hypersecretory phenotype of these two conditions. Although mucus abnormalities are considered a feature of asthma, it is not clear whether these abnormalities result from excessive production of mucus, an intrinsic biochemical abnormality in asthmatic mucus, interactions between mucus and other airway components, or a combination of any or all of these factors. These possibilities are addressed below.

Characteristics of mucus hypersecretion in asthma

Mucus plugging of the airways is a feature of fatal asthma in both adults and children [9•,10•] (Figure 1). Unlike those in COPD, airway plugs of asthmatic patients are difficult to dislodge from the airways [11]. The plugs comprise plasma proteins, DNA, cells and proteoglycans, with mucins being the major gel-forming component [12]. Mucus plugging is not found in all patients dying of asthma; however, incomplete plugs often encrust the airways of asthmatic subjects who have died from causes other than their asthma [9•]. The latter observation indicates that plug formation is a chronic process that progresses to airway occlusion.

Markedly increased amounts of mucus are found throughout the airways of chronic asthmatic patients and in severe fatal asthma [9•,13,14•] (Figure 3). The increased luminal mucus is associated with sputum production, particularly during or just after acute attacks [15]. Increased sputum production is associated with increased mucus secretion, as determined by elevated mucin markers in sputum [16,17]. Several other molecules are also increased in asthmatic sputum, including DNA, lactoferrin, eosinophil cationic protein and plasma proteins (e.g. albumin and fibrinogen) [18]. Thus, increased mucin secretion, plasma exudation and inflammatory cell secretion are associated with mucus hypersecretion and sputum production in asthma.

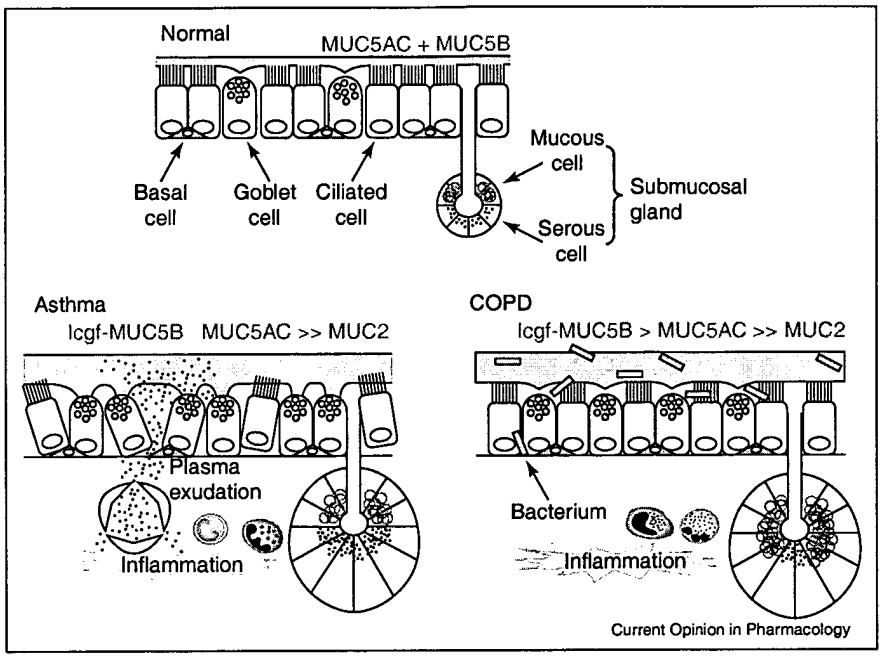
Increased mucin output reflects an increased amount of airway secretory apparatus. Submucosal gland hypertrophy is a pathophysiological feature of asthma, with the size of the glands increased two- to four-fold above controls (Figure 4) [13,14•,19]. However, gland hypertrophy is not common to all patients, even those with sputum production [20]. As a result, inflammation 'score' is a better morphological correlate of airway mucus hypersecretion than is gland size. Thus, although submucosal gland hypertrophy contributes to excess luminal mucus, glands of normal size can hypersecrete, presumably because of the influence of other factors, in particular airway inflammation.

Goblet cell hyperplasia is another pathophysiological feature of asthma, with an increased area and number of goblet cells found throughout the lower airways of patients with asthma [5,21•]. Again, goblet cell hyperplasia is not common to all patients. In a small cohort of Japanese asthmatics, goblet cells were increased markedly in acute severe patients but not in the airways of patients with chronic asthma (Figure 5) [13]. The degree of gland hypertrophy was similar between the two patient groups (Figure 4). The latter observations require confirmation but indicate that, in fatal asthma, airway submucosal gland hypertrophy is a non-specific feature, whereas disproportionate goblet cell hyperplasia is associated with, and possibly contributes to, deaths from asthma.

Mucus abnormalities asthma

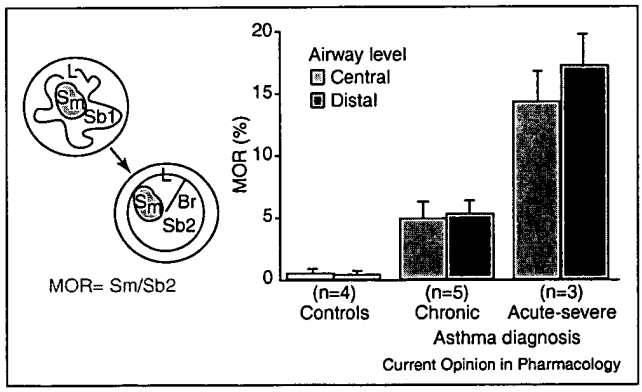
Is there an intrinsic biochemical abnormality of mucus in asthma? The viscosity of asthmatic sputum is greater than that of patients with COPD or bronchiectasis [16,22,23].

Figure 2



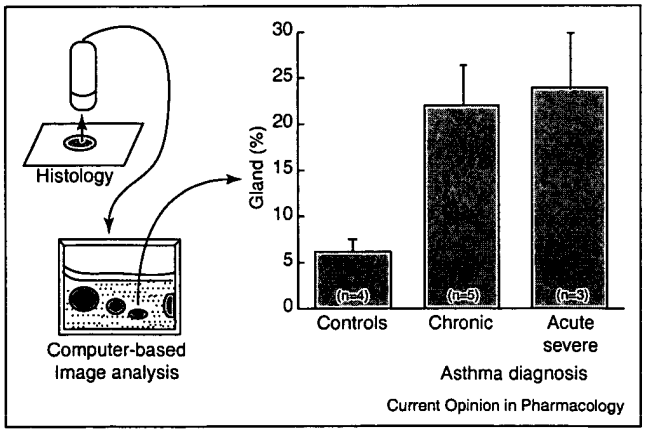
Mucus pathophysiology in asthma and COPD: similarities and differences. In asthmatics, there is increased luminal mucus, a similar or increased ratio of mucin (MUC) 5B (low charge glycoform [lcgf]) to MUC5AC, small amounts of MUC2, epithelial 'fragility', marked goblet cell hyperplasia, submucosal gland hypertrophy (with normal mucous to serous cell ratio), 'tethering' of mucus to goblet cells, and plasma exudation. Airway inflammation involves T lymphocytes and eosinophils. In COPD, there is increased luminal mucus, an increased ratio of lcgf MUC5B to MUC5AC, small amounts of MUC2, goblet cell hyperplasia, submucosal gland hypertrophy (with an increased proportion of mucous to serous cells), and respiratory infection (possibly owing to reduced bacterial enzymatic 'shield' from reduced serous cell number). Pulmonary inflammation involves macrophages and neutrophils. Many of these differences require data from greater numbers of patients.

Figure 3



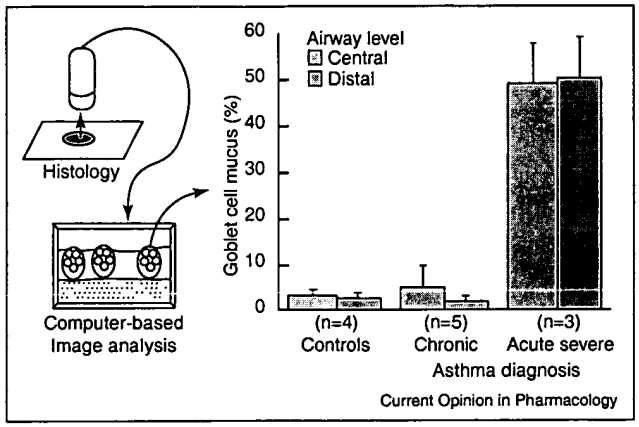
Airway luminal mucus in asthma. Area of luminal mucus was quantified morphometrically in histological sections of autopsied lungs of patients without lung disease (controls), patients with chronic asthma, or patients who had died of a sudden attack. Mucus occupying ratio (MOR) is significantly greater in both central and distal airways of patients who die with a diagnosis of asthma compared with controls. Br, bronchial radius; L, luminal perimeter; S_m, size of stained area of mucus; Sb_1, size of bronchus before computer-based image analysis conversion to a circle, Sb_2. Redrawn using data from [13].

Figure 4



Submucosal gland hypertrophy in asthma. Gland area was quantified morphometrically and expressed as the proportion of gland to bronchial wall in histological sections of autopsied lungs of patients without lung disease (controls), patients with chronic asthma, or patients who had died of a sudden attack. Redrawn using data in [13].

Figure 5



Goblet cell hyperplasia in asthma. The area of stained goblet cell mucin was quantified morphometrically and expressed as the proportion of mucin to total epithelial layer in histological sections of autopsied lungs of patients without lung disease (controls), patients with chronic asthma, or patients who had died of a sudden attack. Redrawn using data in [13].

Mucus plugs from a patient dying of asthma have notably different cross-linking, size, acidity and appearance (assessed by electron microscopy) compared with control mucus [12]. Characteristics of the mucins could explain the solidity of the mucus plugs; the authors concluded that the asthmatic mucus gel was stabilized by non-covalent interactions between extremely large mucins assembled from 'normal' sized subunits. This implies an abnormality in the mucin molecule assembly process. Although confirmatory studies are required, this investigation represents the first reliable demonstration of an intrinsic abnormality in mucus in asthma.

Is the abnormality in mucus caused by the presence, or absence, of a specific mucin species in asthmatic secretions? The MUC5AC gene product and a low charge glycoform of MUC5B are the major mucin species in airway secretions from patients with asthma [7,24•,25,26]. Both mucins are increased above the levels found in secretions from control subjects, in particular the low charge glycoform of MUC5B [24•,26–28]. Accumulating data indicate that in 'irritated' airways, including those in asthma, MUC2 and MUC5B become significantly expressed in goblet cells, in which MUC5B is normally found at low levels and MUC2 not at all [4•]. A correlate of this is that MUC2 gene expression is raised in the airways of asthmatic patients [29]; MUC2 mucin is detected in induced sputum from only one healthy subject out of 15, but was present in sputum from three out of six asthmatic patients [24•]. Studies in more patients are required to confirm if upregulation of MUC2 occurs in asthma. Whether or not incorporation of small quantities of MUC2, an insoluble mucin, in the asthmatic mucus gel has a pathophysiological correlate could then be examined.

Interactions between mucus and other airway components

Airway mucus in asthma is composed of numerous constituents other than mucins (see above). In experimental systems, several of these constituents interact with mucin in a way that is detrimental to airway homeostasis. Increased plasma exudation from the bronchial microvasculature into the airway lumen is a feature of asthma [30]; this plasma increases luminal liquid volume and stimulates mucus secretion, thereby further increasing liquid volume. *In vitro*, plasma albumin and DNA (also found in asthmatic sputum) synergistically increase mucus viscosity. There is little evidence that this happens *in vivo* in asthmatic patients, at least not at the concentrations of albumin and DNA found in asthmatic sputum. However, the combined effect might be sufficient to affect mucus viscosity. Thus, plasma exudation can directly and indirectly increase the amount of airway mucus, with albumin and DNA possibly increasing mucus viscosity.

Another possible interaction might occur between newly secreted mucin and inflammatory cell products. Mucin appears to be 'tethered' to goblet cells in fatal asthma, which is in contrast to patients with chronic bronchitis or control subjects [31]. The favoured explanation of the authors is that, in chronic bronchitis, proteases from neutrophils, the predominant inflammatory cell in the airways in COPD [8], cleave mucins attached to the cell surface of goblet cells. Eosinophils, rather than neutrophils, are the predominant inflammatory cell in asthmatic airways, and certainly predominated in the airways of the patients in the above study [31]. There is no evidence that eosinophil products are able to cleave mucins. Thus, 'tethering' of secreted mucin to goblet cells is specific to asthma, with the lack of neutrophils in the airways of patients dying of asthma contributing to mucus plugging. This hypothesis needs formal investigation.

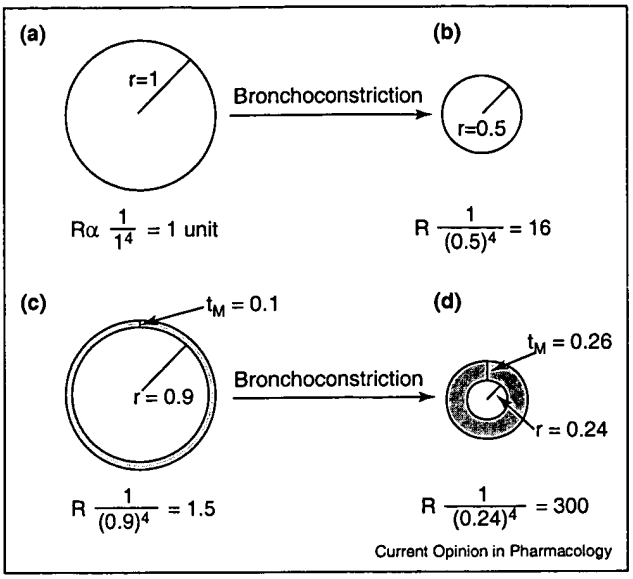
Pathophysiological consequences of mucus hypersecretion and hyperviscosity

The previous section established that there is overproduction of hyperviscous mucus in asthmatic airways. Depending upon how much is present, increased mucus in the airway lumen might not noticeably affect airflow (Figure 6). Larger quantities may still not affect airflow, but might induce cough that contributes markedly to patient morbidity [32]. In asthma, there are two main potential consequences of abnormal airway mucus: airway obstruction and increased airway responsiveness.

Airway obstruction

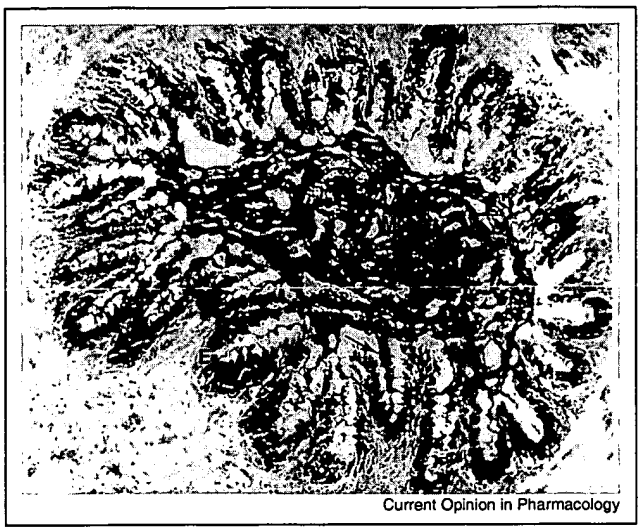
Airway obstruction with mucus and partially formed or complete mucus plugs is a feature of asthma (see above). Airway obstruction by mucus develops as a result of a combination of mucus abnormalities and ciliary dysfunction, leading to reduced mucociliary clearance, mucostasis and plugging. Excess mucus compromises small diameter

Figure 6



Theoretical amplifying effect of luminal mucus on airflow resistance in asthma. **(a)** According to Poiseuille's law, resistance to flow (R) is proportional to the reciprocal of the radius (r) raised to the fourth power. **(b)** Without luminal mucus, bronchoconstriction to reduce the airway radius by half increases airflow resistance 16-fold. **(c)** A small increase in mucus thickness (t_M), which reduces the radius of the airway by only one-tenth, has a negligible effect on airflow in the unconstricted airway (compare with panel a). **(d)** With bronchoconstriction, the same amount of luminal mucus markedly amplifies the airflow resistance of this airway.

Figure 7



Effect of bronchoconstriction and luminal mucus on airway calibre in asthma. Marked bronchoconstriction in an intrapulmonary airway of a patient who died of an acute severe attack has thrown the epithelium (E) into tight, concertina-like folds. In the unconstricted airway (approaching circular in cross section), the comparatively small amount of intraluminal mucus might not have a significant effect on resistance to airflow (see Figure 6). However, the combination of intraluminal mucus and bronchoconstriction has occluded this airway. Arrow, mucus filling interstices between epithelial folds.

airway patency more easily than it does large diameter airways. Luminal liquid also produces an inward force owing to surface tension, further compromising airway patency [33]. Mucus clearance is impaired in all stages of asthma, including patients in remission [34]. Factors other than excess mucus can reduce clearance; these include epithelial damage and shedding (with consequent loss of cilia), goblet cell hyperplasia replacing ciliated cells [5], and the generation of mediators that slow mucociliary clearance directly (e.g. leukotriene D_4).

Obstruction of the airways leads to ventilation/perfusion mismatch [35]. Mucus obstruction in the asthmatic lung is patchy, which diverts ventilation from some alveolar regions to others to produce mismatch. There follows arterial hypoxaemia and stimulation of chemoreceptors, leading to hyperventilation and dyspnoea. Luminal mucus also contributes to increased airway resistance and the work of breathing.

Change in airway responsiveness

A reduction in airway luminal cross-sectional area will amplify any increase in airflow resistance due to bronchoconstriction (Figure 6). This exaggerated response is known as airway hyperresponsiveness, and is a clinical characteristic of asthma [36]. Airway wall thickening, increased surface tension at the air–liquid interface, reduced external support of the airway wall, and increased luminal mucus all reduce airway cross-sectional area. Experimental introduction of small glass beads into the airways of anaesthetized and mechanically ventilated cats to mimic increased luminal mucus leads to marked airway hyperresponsiveness [37]. Even small increases in luminal liquid lead to marked airflow limitation with bronchoconstriction (Figure 7) [38]. However, excessive luminal mucus can have variable effects on airway responsiveness to inhaled spasmogens [39,40]. Responsiveness can either fall or increase, depending upon the pattern of distribution of mucus in the airways, which is usually not homogeneous [41,42]. Mucus accumulation in particular regions of the lungs results in uneven flow distribution. Inhaled bronchoconstrictors are then directed to less resistant airways, thereby amplifying their responsiveness.

Epidemiology of mucus hypersecretion in asthma

The detrimental effects of excess mucus on airway mucociliary clearance, luminal patency and airway reactivity outlined above should have concomitant detrimental effects on the asthmatic patient. Certainly, phlegm production (indicative of airway mucus hypersecretion) is an index of poor asthma control [2]. The greatest risk for increased mortality in asthma is decreased lung function, with a significant proportion of the excess risk

associated with mucus hypersecretion [1]. Some of the decrease in lung function will be caused by luminal mucus. Mucus hypersecretion is also a predictor of an excess decline in lung function, although not in all patients. Thus, in general terms, chronic airway mucus hypersecretion is associated with increased morbidity and mortality in patients with asthma. The following section addresses the issue of the derivation of this increased mucus output.

Generation of the airway mucus hypersecretory phenotype in asthma

Most of the numerous inflammatory mediators produced in asthmatic airways, together with neural mechanisms, can theoretically generate the mucus hypersecretory phenotype in asthma. In experimental studies, these mediators and neural mechanisms increase mucin secretion, induce plasma exudation, upregulate MUC gene expression, increase mucin synthesis and cause goblet cell hyperplasia (summarized in Table 1) [43–45]. Th2 lymphocyte-mediated generation of the mucus hypersecretory phenotype in animal models of asthma, and the pivotal involvement of interleukin (IL)-9 and IL-13 in the induction of goblet cell hyperplasia, are well documented [46–49]. Other inflammatory cells involved include mast cells and neutrophils; both of these infiltrate submucosal glands in the airways of asthmatic patients [14•]. Degranulation of mast cells is associated with increased luminal mucus. Mast cells secrete histamine and proteases, whereas neutrophils secrete elastase. These mediators all have effects on mucus in experimental systems [45]. Most of the above pathways operate through the epidermal growth factor receptor and its tyrosine kinase intracellular signalling cascade [50]. Another key element that appears to be involved in the asthmatic hypersecretory phenotype is the human calcium-activated chloride channel [51,52•]. The $Na^+/K^+/Cl^-$ cotransporter isoform 1 is also associated with mucus hypersecretion in asthma [53]. Thus, there are many possible targets for development of antihypersecretory drugs in asthma.

Pharmacotherapy of mucus pathophysiology in asthma

Airway mucus contributes to morbidity and mortality in many asthmatic patients; consequently, drugs affecting

Table 1

Potential mediators of airway mucus secretion, goblet cell hyperplasia, MUC synthesis/gene expression and plasma exudation in asthma.

Stimulation	Secretion	Hyperplasia	MUC	Plasma exudation
Cytokines				
IL-1β	+	NP	NP	NP
IL-6	+	NP	Yes	NP
IL-9	NP	NP	Yes	NP
IL-13 (IL-4)	+	Yes	Yes	NP
TNFα	++	Yes[a]	Yes[a]	NP
Gases				
Irritant gases (e.g. cigarette smoke)	++	Yes	Yes	+
Nitric oxide	-ve/+	NP	NP	+
Reactive oxygen species	0/+	NP	NP	+
Inflammatory mediators				
Bradykinin	+	NP	NP	++
Cysteinyl leukotrienes	++	NP	NP	++
Endothelin	0/+	NP	NP	+
Histamine	+	NP	NP	++
PAF	+	Yes[a]	Yes[a]	+++
Prostaglandins	0/+	NP	NP	0/+
Proteinases	+++	Yes	NP	NP
Purine nucleotides	++	NP	NP	NP
Neural pathways				
Cholinergic nerves	++	NP	NP	0
Cholinoceptor agonists	++	Yes	NP	0
Nicotine	++	Yes	NP	++
Tachykininergic nerves	+	NP	NP	++
Substance P	++	NP	NP	+++
Neurokinin A	+	NP	NP	++
Miscellaneous				
EGF (+ TNFα)	NP	Yes	Yes	NP
Sensitisation followed by challenge	+	Yes	Yes	++

[a]Effect only observed with PAF and TNFα in combination. +++, highly potent; ++, marked effect; +, lesser effect; 0, minimal effect; EGF, epidermal growth factor; NP, effect not published; PAF, platelet activating factor; TNFα, tumour necrosis factor-α.

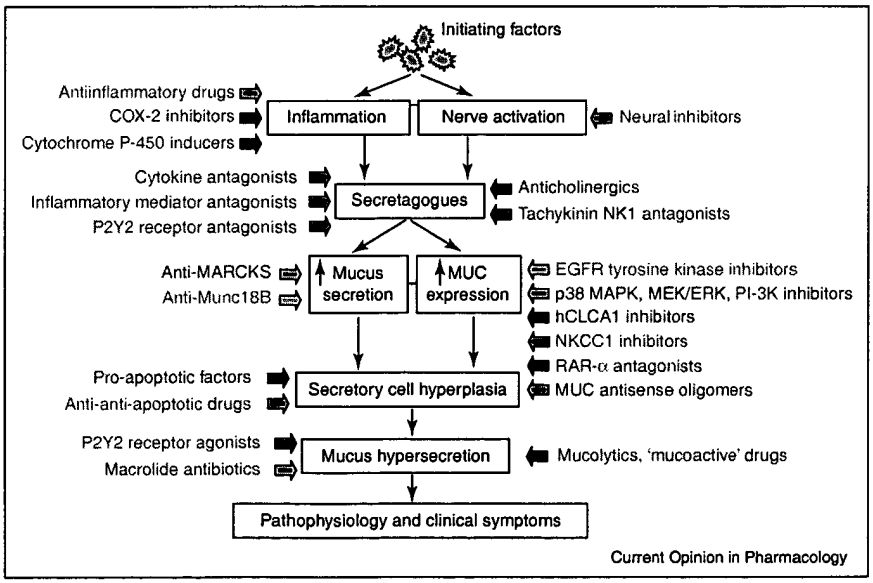
Table 2

Theoretical objectives for effective pharmacotherapy of airway hypersecretory pathophysiology in asthma.

Overall objective	Specific objectives
Facilitate mucus clearance (short-term relief of symptoms)	Inhibit mucin secretion Inhibit plasma exudation Reduce mucus viscosity (increase elasticity) Increase ciliary function Induce cough Facilitate release of 'tethered' goblet cell mucin
Reverse hypersecretory phenotype (long-term benefit)	Treat airway inflammation Reduce goblet cell number Reduce submucosal gland size Inhibit increased production of low charge glycoform of MUC5B Inhibit production of MUC2 Reverse increased MUC5B:MUC5AC ratio

the hypersecretory component of asthma should be beneficial in these patients. Asthma has specific trigger factors, its own 'profile' of pulmonary inflammation and its own mucus hypersecretory phenotype (Figure 2); therefore, specific drugs might be required to fulfil the objectives for treatment of hypersecretion in asthma (Table 2). Pharmacotherapy of airway mucus hypersecretion in asthma has been discussed in detail recently [45] and is summarized herein (Figure 8; Table 3). Essentially, pharmacotherapy can be divided into two sections: firstly, anti-inflammatory treatment of airway inflammation, probably the most beneficial therapy overall; and secondly, therapies directed specifically at different levels of the pathophysiological 'cascade', from secretagogues to clinical symptoms (Figure 8). Many potential therapies are undergoing intensive investigation; for example, myristoylated alanine-rich C kinase substrate (MARCKS) is fundamental to goblet cell mucin exocytosis [54]. A synthetic peptide corresponding to the N-terminal domain of MARCKS inhibits airway mucin secretion in a mouse model of asthma [55•]. However, with the exception of glucocorticosteroids, it is unlikely that any single class of compound will provide comprehensive treatment of mucus hypersecretion in asthma.

Figure 8



Pharmacotherapy of airway mucus hypersecretion in asthma. The pathophysiological 'cascade' from initiating factors to clinical symptoms can be accessed at different levels by 'antihypersecretory' pharmacotherapeutic compounds. The precise site(s) of action of many compounds is unclear, and some compounds might act at more than one site. hCLCA, human calcium-activated chloride channel; COX, cyclooxygenase; EGFR, epidermal growth factor receptor; ERK, extracellular signal-regulated kinase; MEK, mitogen-activated protein kinase kinase; MUC, mucin (gene); NKCC, Na^+-K^+-Cl^- cotransporter; PI3K, phosphatidylinositol 3-kinase; RAR, retinoic acid receptor.

Table 3

Potential therapeutic targets and inhibitors of airway mucus hypersecretion in asthma.

Target	Inhibitors
Airway inflammation	Glucocorticosteroids (e.g. ciclesonide), PDE4 inhibitors (e.g. cilomilast, roflumilast), suplatast tosilate, cytokine/chemokine blockers (monoclonal antibodies, soluble receptors, small molecule inhibitors and receptor antagonists), macrolide antibiotics (erythromycin, flurythromycin), iNOS inhibitors (e.g. GW273629, L-NIL), COX-2 inhibitors (e.g. rofcoxib, celecoxib), cytochrome P-450 inducers (e.g. benzafibrate), HO inducers (see Update)
Mucus properties	
Thickened mucus	Mucolytic drugs (e.g. *N*-acetylcysteine, nacystelyn)
$P2Y_2$ receptors	Selective agonists for mucus hydration (e.g. INS37217)
Goblet cell hyperplasia	
Bcl-2	Antisense oligonucleotides (e.g. G3139 [Genasense, oblimersen]), Bax mimetics
hCLCA1	Talniflumate
EGFR tyrosine kinase	AG1478, BIBX1522, ZD1839 (Iressa)
ERK	MEK inhibitors (e.g. PD98059, U0126)
MUC gene expression	18-mer MUC antisense oligonucleotide
NKCC1	Bumetanide
p38 MAPK	p38 MAPK inhibitors (e.g. SB 203580)
PI3K	PI3K inhibitors (e.g. LY-294002)
Inflammatory mediators	
Bradykinin (B_2 receptors)	Icatibant
Endothelin-1 (ET_A receptors)	Bosentan
Cysteinyl leukotrienes (Cys-LT_1 receptors)	Montelukast, zafirlukast
Mast cell tryptase	APC-366, BABIM
Neutrophil elastase	Elastase inhibitors (e.g. batimastat, suramin and macrolide antibiotics such as erythromycin and flurythromycin)
PAF	Apafant, modipafant
$P2Y_2$ purinoceptors	$P2Y_2$ antagonists (none yet available)
Mucin exocytosis	
MARCKS	MARCKS inhibitors (e.g. MANS peptide)
Munc-18B	Munc-18B inhibitors (antisense oligomer)
Neural pathways	
Nerve activation	VR-1 receptor antagonists (e.g. anandamide, capsazepine)
Neurotransmitter release	BK_{Ca} channel activators (e.g. NS 1619), CB_2 receptor agonists (e.g. AM1241, SR144528)
Muscarinic (M_3) receptors	Anticholinergics (e.g. ipratropium bromide, tiotropium)
Tachykinin NK_1 receptors	Tachykinin NK_1 receptor antagonists (e.g. CP99,994, RP67580, nolpitantium; dual NK_1/NK_2 and triple $NK_1/NK_2/NK_3$ antagonists)

BABIM, bis(5-amidino-2-benzimidazolyl)methane; CB, cannabinoid; CLCA, calcium-activated calcium channel; COX, cyclooxygenase; EGFR, epidermal growth factor receptor; ERK, extracellular signal-regulated kinase; HO, heme oxygenase; iNOS, inducible nitric oxide synthase; MAPK, mitogen-activated protein kinase; MEK, MAPK kinase; MUC, mucin; NK, neurokinin; NKCC, $Na^+/K^+/Cl^-$ cotransporter; PAF, platelet activating factor; PDE, phosphodiesterase; PI3K, phosphatidylinositol 3-kinase; VR, vanilloid receptor.

Conclusions

Airway mucus hypersecretion and the pathophysiological changes that accompany it are features of many patients with asthma. The impact of airway hypersecretion on morbidity and mortality is now more fully understood, even though it can often be limited to certain groups of patients. Nevertheless, it is important to develop drugs that inhibit mucus hypersecretion in susceptible patients. Before addressing these issues, more information is required on mucus physiology and pathophysiology, particularly concerning the biochemical and biophysical nature of airway mucins in healthy subjects. Whether or not there is an intrinsic abnormality of mucus in asthma, and whether any abnormality is specific for asthma, requires confirmation. The factors that regulate MUC gene expression in health and disease, and the relationship between this regulation and development of an asthma-specific hypersecretory phenotype, need to be determined. The above information could then be used to delineate therapeutic targets which, in turn, should lead to rational design of anti-hypersecretory drugs for treatment of airway mucus hypersecretion in asthma.

Update

Induction of heme oxygenase, the enzyme that degrades heme, by repeated administrations of hemin demonstrates anti-inflammatory activity in a mouse model of allergic asthma, including inhibition of airway mucus hypersecretion (inhibition of upregulated MUC5AC gene expression and of increased periodic acid-Schiff staining of mucus) [56]. The inhibitory effects of hemin were

reversed by the heme oxygenase inhibitor tin protoporphyrin-IX.

References and recommended reading

Papers of particular interest, published within the annual period of review, have been highlighted as:

- • of special interest
- •• of outstanding interest

1. Vestbo J: **Epidemiological studies in mucus hypersecretion.** *Novartis Found Symp* 2002, **248**:3-12.

2. National Institutes of Health: **Global Initiative for Asthma: pocket guide for asthma management and prevention.** Bethesda: National Institutes of Health, National Heart, Lung and Blood Institute; 2002, Publication no. 02-3659.

3. • Knowles MR, Boucher RC: **Mucus clearance as a primary innate defense mechanism for mammalian airways.** *J Clin Invest* 2002, **109**:571-577.

Intelligent and thoughtful review of the factors involved in effective mucus clearance in human lower airways, with a consideration of two alternate views of innate defence mechanisms in the airways.

4. • Davies JR, Herrmann A, Russell W, Svitacheva N, Wickström C, Carlstedt I: **Respiratory tract mucins: structure and expression patterns.** In *Mucus Hypersecretion in Respiratory Disease.* Chichester: John Wiley & Sons; 2002:76-88.

Overview of airway mucins by one of the world's leading groups of mucin biochemists, including new information on endogenous proteolytic processing of mucins.

5. Rogers DF: **The airway goblet cell.** *Int J Biochem Cell Biol* 2003, **35**:1-6.

6. Finkbeiner WE: **Physiology and pathology of tracheobronchial glands.** *Respir Physiol* 1999, **118**:77-83.

7. Rogers DF: **Mucus pathophysiology in COPD: differences to asthma, and pharmacotherapy.** *Monaldi Arch Chest Dis* 2000, **55**:324-332.

8. Sutherland ER, Martin RJ: **Airway inflammation in chronic obstructive pulmonary disease: comparisons with asthma.** *J Allergy Clin Immunol* 2003, **112**:819-827.

9. • Sidebotham HJ, Roche WR: **Asthma deaths; persistent and preventable mortality.** *Histopathology* 2003, **43**:105-117.

Thorough review (including many figures) of fatal asthma, with an emphasis on the pathology and histopathology of the airways of patients dying of asthma.

10. • Rogers DF: **Pulmonary mucus: pediatric perspective.** *Pediatr Pulmonol* 2003, **36**:178-188.

Review of airway mucus physiology and pathophysiology in children, in contrast to most reviews, which refer to adult airway disease (e.g. [7]).

11. Dunnill MS, Massarella GR, Anderson JA: **A comparison of the quantitative anatomy of the bronchi in normal subjects, in status asthmaticus, in chronic bronchitis, and in emphysema.** *Thorax* 1969, **24**:176-179.

12. Sheehan JK, Richardson PS, Fung DC, Howard M, Thornton DJ: **Analysis of respiratory mucus glycoproteins in asthma: a detailed study from a patient who died in status asthmaticus.** *Am J Respir Cell Mol Biol* 1995, **13**:748-756.

13. Aikawa T, Shimura S, Sasaki H, Ebina M, Takishima T: **Marked goblet cell hyperplasia with mucus accumulation in the airways of patients who died of severe acute asthma attack.** *Chest* 1992, **101**:916-921.

14. • Carroll NG, Mutavdzic S, James AL: **Increased mast cells and neutrophils in submucosal mucous glands and mucus plugging in patients with asthma.** *Thorax* 2002, **57**:677-682.

Original paper that makes the interesting observation that mast cells and neutrophils infiltrate the stromal tissue of submucosal glands in non-fatal and fatal asthma. The authors conclude that mast cell degranulation, rather than neutrophil accumulation, might contribute to mucus hypersecretion in fatal asthma.

15. Openshaw PJ, Turner-Warwick M: **Observations on sputum production in patients with variable airflow obstruction; implications for the diagnosis of asthma and chronic bronchitis.** *Respir Med* 1989, **83**:25-31.

16. Lopez-Vidriero MT, Reid L: **Chemical markers of mucous and serum glycoproteins and their relation to viscosity in mucoid and purulent sputum from various hypersecretory diseases.** *Am Rev Respir Dis* 1978, **117**:465-477.

17. Fahy JV, Steiger DJ, Liu J, Basbaum CB, Finkbeiner WE, Boushey HA: **Markers of mucus secretion and DNA levels in induced sputum from asthmatic and from healthy subjects.** *Am Rev Respir Dis* 1993, **147**:1132-1137.

18. Fahy JV, Liu J, Wong H, Boushey HA: **Cellular and biochemical analysis of induced sputum from asthmatic and from healthy subjects.** *Am Rev Respir Dis* 1993, **147**:1126-1131.

19. Carroll N, Elliot J, Morton A, James A: **The structure of large and small airways in nonfatal and fatal asthma.** *Am Rev Respir Dis* 1993, **147**:405-410.

20. Jeffery P, Zhu J: **Mucin-producing elements and inflammatory cells.** *Novartis Found Symp* 2002, **248**:51-68.

21. • Fahy JV: **Goblet cell and mucin gene abnormalities in asthma.** *Chest* 2002, **122**:320S-326S.

Interesting and thoughtful review of goblet cell pathophysiology in asthma, including expanded discussion of the author's own published work on goblet cells in asthma.

22. Charman J, Reid L: **Sputum viscosity in chronic bronchitis, bronchiectasis, asthma and cystic fibrosis.** *Biorheology* 1972, **9**:185-199.

23. Shimura S, Sasaki T, Sasaki H, Takishima T, Umeya K: **Viscoelastic properties of bronchorrhoea sputum in bronchial asthmatics.** *Biorheology* 1988, **25**:173-179.

24. • Kirkham S, Sheehan JK, Knight D, Richardson PS, Thornton DJ: **Heterogeneity of airways mucus: variations in the amounts and glycoforms of the major oligomeric mucins MUC5AC and MUC5B.** *Biochem J* 2002, **361**:537-546.

Original paper from one of the world's leading groups of mucin biochemists that describes a thorough analysis of airway mucins, and demonstrates fundamental differences between mucins from normal subjects and mucins from patients with respiratory disease (e.g. an increase in the low-charge glycoform of MUC5B in disease).

25. Thornton DJ, Howard M, Khan N, Sheehan JK: **Identification of two glycoforms of the MUC5B mucin in human respiratory mucus. Evidence for a cysteine-rich sequence repeated within the molecule.** *J Biol Chem* 1997, **272**:9561-9566.

26. Sheehan JK, Howard M, Richardson PS, Longwill T, Thornton DJ: **Physical characterization of a low-charge glycoform of the MUC5B mucin comprising the gel-phase of an asthmatic respiratory mucous plug.** *Biochem J* 1999, **338**:507-513.

27. Hovenberg HW, Davies JR, Herrmann A, Linden CJ, Carlstedt I: **MUC5AC, but not MUC2, is a prominent mucin in respiratory secretions.** *Glycoconj J* 1996, **13**:839-847.

28. Wickstrom C, Davies JR, Eriksen GV, Veerman EC, Carlstedt I: **MUC5B is a major gel-forming, oligomeric mucin from human salivary gland, respiratory tract and endocervix: identification of glycoforms and C-terminal cleavage.** *Biochem J* 1998, **334**:685-693.

29. Ordonez CL, Khashayar R, Wong HH, Ferrando R, Wu R, Hyde DM, Hotchkiss JA, Zhang Y, Novikov A, Dolganov G, Fahy JV: **Mild and moderate asthma is associated with airway goblet cell hyperplasia and abnormalities in mucin gene expression.** *Am J Respir Crit Care Med* 2001, **163**:517-523.

30. Rogers DF, Evans TW: **Plasma exudation and oedema in asthma.** *Br Med Bull* 1992, **48**:120-134.

31. Shimura S, Andoh Y, Haraguchi M, Shirato K: **Continuity of airway goblet cells and intraluminal mucus in the airways of patients with bronchial asthma.** *Eur Respir J* 1996, **9**:1395-1401.

32. Dicpinigaitis PV: **Cough. 4: Cough in asthma and eosinophilic bronchitis.** *Thorax* 2004, **59**:71-72.

33. O'Riordan TG, Zwang J, Smaldone GC: **Mucociliary clearance in adult asthma.** *Am Rev Respir Dis* 1992, **146**:598-603.

34. Del Donno M, Bittesnich D, Chetta A, Olivieri D, Lopez-Vidriero MT: **The effect of inflammation on mucociliary clearance in asthma: an overview.** *Chest* 2000, **118**:1142-1149.

35. Wagner PD, Hedenstierna G, Rodriguez-Roisin R: **Gas exchange, expiratory flow obstruction and the clinical spectrum of asthma.** *Eur Respir J* 1996, **9**:1278-1282.

36. Pare PD: **Airway hyperresponsiveness in asthma: geometry is not everything!** *Am J Respir Crit Care Med* 2003, **168**:913-914.

37. Suzuki T, Inoue H, Lin J-T, Takishima T: **Intraluminal space occupying substance induces airway hyperresponsiveness.** *Am Rev Respir Dis* 1992, **145(suppl)**:A50.

38. Yager D, Shore S, Drazen JM: **Airway luminal liquid. Sources and role as an amplifier of bronchoconstriction.** *Am Rev Respir Dis* 1991, **143**:S52-S54.

39. King M, Kelly S, Cosio M: **Alteration of airway reactivity by mucus.** *Respir Physiol* 1985, **62**:47-59.

40. Kim CS, Eldridge MA, Wanner A: **Airway responsiveness to inhaled and intravenous carbachol in sheep: effect of airway mucus.** *J Appl Physiol* 1988, **65**:2744-2751.

41. Kim CS, Eldridge MA: **Aerosol deposition in the airway model with excessive mucus secretions.** *J Appl Physiol* 1985, **59**:1766-1772.

42. Kim CS, Abraham WM, Garcia L, Sackner MA: **Enhanced aerosol deposition in the lung with mild airways obstruction.** *Am Rev Respir Dis* 1989, **139**:422-426.

43. Jackson AD: **Airway goblet-cell mucus secretion.** *Trends Pharmacol Sci* 2001, **22**:39-45.

44. Rogers DF: **Pharmacological regulation of the neuronal control of airway mucus secretion.** *Curr Opin Pharmacol* 2002, **2**:249-255.

45. Rogers DF: **Airway hypersecretion in allergic rhinitis and asthma: new pharmacotherapy.** *Curr Allergy Asthma Rep* 2003, **3**:238-248.

46. Wills-Karp M: **Trophic slime, allergic slime.** *Am J Respir Cell Mol Biol* 2000, **22**:637-639.

47. Reader JR, Hyde DM, Schelegle ES, Aldrich MC, Stoddard AM, McLane MP, Levitt RC, Tepper JS: **Interleukin-9 induces mucous cell metaplasia independent of inflammation.** *Am J Respir Cell Mol Biol* 2003, **28**:664-672.

48. Kuperman DA, Huang X, Koth LL, Chang GH, Dolganov GM, Zhu Z, Elias JA, Sheppard D, Erle DJ: **Direct effects of interleukin-13 on epithelial cells cause airway hyperreactivity and mucus overproduction in asthma.** *Nat Med* 2002, **8**:885-889.

49. Atherton HC, Jones G, Danahay H: **IL-13-induced changes in the goblet cell density of human bronchial epithelial cell cultures: MAP kinase and phosphatidylinositol 3-kinase regulation.** *Am J Physiol Lung Cell Mol Physiol* 2003, **285**:L730-L739.

50. Nadel JA, Burgel PR: **The role of epidermal growth factor in mucus production.** *Curr Opin Pharmacol* 2001, **1**:254-258.

51. Hoshino M, Morita S, Iwashita H, Sagiya Y, Nagi T, Nakanishi A, Ashida Y, Nishimura O, Fujisawa Y, Fujino M: **Increased expression of the human $Ca2^{+}$-activated Cl^{-} channel 1 (CaCC1) gene in the asthmatic airway.** *Am J Respir Crit Care Med* 2002, **165**:1132-1136.

52. • Toda M, Tulic MK, Levitt RC, Hamid Q: **A calcium-activated chloride channel (HCLCA1) is strongly related to IL-9 expression and mucus production in bronchial epithelium of patients with asthma.** *J Allergy Clin Immunol* 2002, **109**:246-250.
Building upon evidence in animal models of asthma that calcium-activated chloride channels are involved in generation of a hypersecretory phenotype, this original research paper demonstrates that the human variant of these channels is upregulated in asthma and is associated with the mucus hypersecretory phenotype.

53. Dolganov GM, Woodruff PG, Novikov AA, Zhang Y, Ferrando RE, Szubin R, Fahy JV: **A novel method of gene transcript profiling in airway biopsy homogenates reveals increased expression of a Na^{+}-K^{+}-Cl^{-} cotransporter (NKCC1) in asthmatic subjects.** *Genome Res* 2001, **11**:1473-1483.

54. Li Y, Martin LD, Spizz G, Adler KB: **MARCKS protein is a key molecule regulating mucin secretion by human airway epithelial cells *in vitro*.** *J Biol Chem* 2001, **276**:40982-40990.

55. • Singer M, Martin LD, Vargaftig BB, Park J, Gruber AD, Li Y, Adler KB: **A MARCKS-related peptide blocks mucus hypersecretion in a mouse model of asthma.** *Nat Med* 2004, **10**:193-196.
Original article demonstrating that an N-terminal 24-amino-acid fragment of MARCKS (MANS peptide) blocks mucin exocytosis in a mouse model of allergic asthma.

56. Almolki A, Taille C, Martin GF, Jose PJ, Zedda C, Conti M, Megret J, Henin D, Aubier M, Boczkowski J: **Heme oxygenase attenuates allergen-induced airway inflammation and hyperreactivity in guinea pigs.** *Am J Physiol Lung Cell Mol Physiol* 2004, [Epub ahead of print].

Attorney Docket No. 5051-451IP PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Inventor: Li et al. Confirmation No.: 8515
Serial No.: 09/914,020 Art Unit: 1633
Filed: December 31, 2001 Examiner: J. Epps-Ford
For: *Methods and Compositions for Altering Mucus Secretion*

Date: June 7, 2006

Mailstop Amendment
Commissioner for Patents
PO Box 1450
Alexandria, VA 22313-1450

ATTACHMENT C

Exhibit 6

Li et al. (2001) *"MARCKS Protein is a Key Molecule Regulating Mucin Secretion by Human Airway Epithelial Cells in Vitro"* J. Biol. Chem., Vol 276(44), pp.40982-40990.

THE JOURNAL OF BIOLOGICAL CHEMISTRY Vol. 276, No. 44, Issue of November 2, pp. 40982–40990, 2001
Printed in U.S.A.

MARCKS Protein Is a Key Molecule Regulating Mucin Secretion by Human Airway Epithelial Cells *in Vitro**

Received for publication, June 18, 2001, and in revised form, August 30, 2001
Published, JBC Papers in Press, August 31, 2001, DOI 10.1074/jbc.M105614200

Yuehua Li‡§, Linda D. Martin‡, Gwendolyn Spizz¶, and Kenneth B. Adler‡‖

From the ‡Department of Anatomy, Physiological Sciences and Radiology, College of Veterinary Medicine, North Carolina State University, Raleigh, North Carolina 27606 and ¶Laboratory of Signal Transduction, NIEHS, National Institutes of Health, Research Triangle Park, North Carolina 27709

Hypersecretion of airway mucin characterizes numerous respiratory diseases. Although diverse pathological stimuli can provoke exocytotic release of mucin from secretory cells of the airway epithelium, mechanisms involved remain obscure. This report describes a new paradigm for the intracellular signaling mechanism regulating airway mucin secretion. Direct evidence is provided that the myristoylated alanine-rich C kinase substrate (MARCKS) is a central regulatory molecule linking secretagogue stimulation at the cell surface to mucin granule release by differentiated normal human bronchial epithelial cells *in vitro*. Down-regulation of MARCKS expression or disruption of MARCKS function in these cells inhibits the secretory response to subsequent stimulation. The intracellular mechanism controlling this secretory process involves cooperative action of two separate protein kinases, protein kinase C and cGMP-dependent protein kinase. Upon stimulation, activated protein kinase C phosphorylates MARCKS, causing translocation of MARCKS from the plasma membrane to the cytoplasm, where it is then dephosphorylated by a protein phosphatase 2A that is activated by cGMP-dependent protein kinase, and associates with both actin and myosin. Dephosphorylated cytoplasmic MARCKS would also be free to interact with mucin granule membranes and thus could link granules to the contractile cytoskeleton, mediating their movement to the cell periphery and subsequent exocytosis. These findings suggest several novel intracellular targets for pharmacological intervention in disorders involving aberrant secretion of respiratory mucin and may relate to other lesions involving exocytosis of membrane-bound granules in various cells and tissues.

Mammalian airways are lined by a thin layer of mucus produced and secreted by airway epithelial (goblet) cells and submucosal glands. In diseases such as asthma, chronic bronchitis, and cystic fibrosis, hypersecretion of mucus is a common lesion. Excess mucus can contribute to obstruction, susceptibility to infection, and even to destruction of airway walls and contiguous tissues. The major components of mucus are mucin glycoproteins synthesized by secretory cells and stored within cytoplasmic membrane-bound granules. Upon appropriate stimulation, these granules are released via an exocytotic process in which the granules translocate to the cell periphery where the granule membranes fuse with the plasma membrane, allowing for luminal secretion of the contents.

Despite the obvious pathophysiological importance of this process, intracellular signaling mechanisms linking stimulation at the cell surface to mucin granule release have not been elucidated. It is known that a wide variety of agents and inflammatory/humoral mediators can provoke mucin secretion. These include cholinergic agonists, lipid mediators, oxidants, cytokines, neuropeptides, ATP and UTP, bacterial products, neutrophil elastase, and inhaled pollutants (reviewed in Refs. 1 and 2). Interestingly, many of these mucin secretagogues are also known to activate several protein kinases, and studies examining the regulation of excess secretion of mucin by airway epithelial cells from various species have consistently implicated involvement of either protein kinase C (PKC)[1] (3–6) or cGMP-dependent protein kinase (PKG) (7) in the secretory process. However, coordinated interactions or "cross-talk" between these two protein kinases in regulation of mucin secretion have not been demonstrated, nor have signaling events downstream of protein kinase activation that ultimately lead to the exocytotic release of mucin granules been elucidated.

Previously, we reported (8) development of a procedure to culture normal human bronchial epithelial (NHBE) cells in an air/liquid interface system in which the cells differentiate to a heterogeneous population containing secretory (goblet), ciliated, and basal cells that mimic their *in vivo* appearance and function. These cell cultures provide an ideal *in vitro* model system to study mechanisms regulating mucin secretion from human airway epithelium.

This report presents direct evidence demonstrating that the myristoylated alanine-rich C kinase substrate (MARCKS), a widely distributed PKC substrate for which a specific biological function has yet to be identified, is a key regulatory molecule mediating mucin granule release by NHBE cells. Secretion of mucin from these cells is maximized by activation of both PKC and PKG, and MARCKS serves as the point of convergence for coordinating the actions of these two protein kinases to control mucin granule release. The mechanism appears to involve PKC-

* This work was supported by Grant RO1 HL39862 from the National Institutes of Health and a grant from the state of North Carolina. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ Performed portions of this work in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Comparative Biomedical Sciences.

‖ To whom correspondence should be addressed: North Carolina State University, College of Veterinary Medicine, 4700 Hillsborough St., Raleigh, NC 27606. Tel.: 919-513-1348; Fax: 919-513-6465; E-mail: Kenneth_Adler@ncsu.edu.

[1] PKC, protein kinase C; PKG, cGMP-dependent protein kinase; PKA, protein kinase A; MANS, myristoylated N-terminal sequence; PMA, phorbol 12-myristate 13-acetate; PSD, phosphorylation site domain; 8-Br-GMP, 8-bromo-cyclic GMP; RNS, random N-terminal sequence; MARCKS, myristoylated alanine-rich C kinase substrate; NHBE, normal human bronchial epithelial; PP2A, protein phosphatase 2A; ELISA, enzyme-linked immunosorbent assay.

dependent phosphorylation of MARCKS, which releases MARCKS from the plasma membrane into the cytoplasm, where it is in turn dephosphorylated by a protein phosphatase 2A (PP2A) that is activated by PKG. This dephosphorylation would allow MARCKS to regain its membrane-binding capability (9–11), enabling its attachment to membranes of cytoplasmic mucin granules. In addition, MARCKS interacts with actin and myosin in the cytoplasm and thus could tether the granules to the cellular contractile apparatus, mediating subsequent granule movement and exocytosis.

EXPERIMENTAL PROCEDURES

NHBE Cell Culture—Expansion, cryopreservation, and culture of NHBE cells in the air/liquid interface were performed as described previously (8). Briefly, NHBE cells (Clonetics, San Diego, CA) were seeded in vented T75 tissue culture flasks (500 cells/cm^2) and cultured until cells reached 75–80% confluence. Cells were then dissociated by trypsin/EDTA and frozen as passage-2. Air/liquid interface culture was initiated by seeding passage-2 cells (2×10^4 cells/cm^2) in Transwell® clear culture inserts (Costar, Cambridge, MA) that were thinly coated with rat tail collagen, type I (Collaborative Biomedical, Bedford, MA). Cells were cultured submerged in medium in a humidified 95% air, 5% CO_2 environment for 5–7 days until nearly confluent. At that time, the air/liquid interface was created by removing the apical medium and feeding cells basalaterally. Medium was renewed daily thereafter. Cells were cultured for an additional 14 days to allow full differentiation.

Measurement of Mucin Secretion by ELISA—Before collection of "base line" and "test" mucin samples, the accumulated mucus at the apical surface of the cells was removed by washing with phosphate-buffered saline, pH 7.2. To collect the base-line secretion, cells were incubated with medium alone, and secreted mucin in the apical medium was collected and reserved. Cells were rested for 24 h and then exposed to medium containing the selected stimulatory and/or inhibitory reagents (or appropriate controls), after which secreted mucin was collected and reserved as the test sample. Incubation times for the base line and the test were the same but varied depending on the test reagent utilized. Both base line and test secretions were analyzed by ELISA using an antibody capture method described previously (12). The primary antibody for this assay was 17Q2 (Babco, Richmond, CA), a monoclonal antibody that reacts specifically with a carbohydrate epitope on human airway mucins (13). The ratio of test/base-line mucin, similar to a "secretory index" reported previously (14), was used to quantify mucin secretion, allowing each culture dish to serve as its own control and thus minimizing deviation caused by variability among culture wells. Levels of mucin secretion were reported as percentage of the medium control.

Radiolabeled Immunoprecipitation Assay—When labeling with [^{32}P]phosphate, cells were preincubated for 2 h in phosphate-free Dulbecco's modified Eagle's medium containing 0.2% bovine serum albumin and then labeled with 0.1 mCi/ml [^{32}P]orthophosphate (9000 Ci/mmol, PerkinElmer Life Sciences) for 2 h. For labeling with [^{3}H]myristic acid or ^{3}H-amino acids, cells were incubated overnight in medium containing 50 μCi/ml [^{3}H]myristic acid (49 Ci/mmol, PerkinElmer Life Sciences) or 0.2 mCi/ml [^{3}H]leucine (159 Ci/mmol, PerkinElmer Life Sciences) plus 0.4 mCi/ml [^{3}H]proline (100 Ci/mmol, PerkinElmer Life Sciences). Following labeling, cells were exposed to stimulatory reagents for 5 min. When an inhibitor was used, cells were preincubated with the inhibitor for 15 min prior to stimulation. At the end of the treatments, cells were lysed in a buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 10% glycerol, 1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine, 10 μg/ml pepstatin A, and 10 μg/ml leupeptin. The radiolabeling efficiency in each culture was determined by trichloroacetic acid precipitation and scintillation counting. Immunoprecipitation of MARCKS protein was carried out according to the method of Spizz and Blackshear (15) using cell lysates containing equal counts/min. Precipitated proteins were resolved by 8% SDS-polyacrylamide gel electrophoresis and visualized by autoradiography. Anti-human MARCKS antibody (2F12) and nonimmune control antibody (6F6) used in this assay were provided by Dr. Perry Blackshear (NIEHS, Research Triangle Park, NC).

To assess MARCKS or MARCKS-associated protein complexes in different subcellular fractions, radiolabeled and treated cells were scraped into a homogenization buffer (50 mM Tris-HCl (pH 7.5), 10 mM NaCl, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine, 10 μg/ml pepstatin A, 10 μg/ml leupeptin) and then disrupted by nitrogen cavitation (800 pounds/square inch for 20 min at 4 °C). Cell lysates were centrifuged at 600 × *g* for 10 min at 4 °C to remove nuclei and unbroken cells. Post-nuclear supernatants were separated into membrane and cytosol fractions by ultracentrifugation at 400,000 × *g* for 30 min at 4 °C. The membrane pellet was solubilized in the lysis buffer by sonication. Immunoprecipitation was then carried out as described above.

MARCKS-related Peptides—Both the myristoylated N-terminal sequence (MANS) and the random N-terminal sequence (RNS) peptides were synthesized at Genemed Synthesis, Inc. (San Francisco, CA), then purified by high pressure liquid chromatography (>95% pure), and confirmed by mass spectroscopy with each showing one single peak with an appropriate molecular mass. The MANS peptide consisted of sequence identical to the first 24 amino acids of MARCKS, *i.e.* the myristoylated N-terminal region that mediates MARCKS insertion into membranes (9–11), MA-GAQFSKTAAKGEAAAERPGEAAVA (where MA = N-terminal myristate chain). The corresponding control peptide (RNS) contained the same amino acid composition as the MANS but arranged in random order, MA-GTAPAAEGAGAEVKRASAEAKQAF. The presence of the hydrophobic myristate moiety in these synthetic peptides enhances their permeability to the plasma membranes, enabling the peptides to be taken up readily by cells. To determine the effects of these peptides on mucin secretion, cells were preincubated with the peptides for 15 min prior to addition of secretagogues, and mucin secretion was then measured by ELISA.

Antisense Oligonucleotides—MARCKS antisense oligonucleotide and its corresponding control oligonucleotide were synthesized at Biognostik GmbH (Gottingen, Germany). NHBE cells were treated with 5 μM antisense or control oligonucleotide apically for 3 days (in the presence of 2 μg/ml lipofectin for the first 24 h). Cells were then incubated with secretagogues, and mucin secretion was measured by ELISA. Total RNA and protein were isolated from treated cells. MARCKS mRNA was assessed by Northern hybridization according to conventional procedures using human MARCKS cDNA (provided by Dr. Perry Blackshear, NIEHS, Research Triangle Park, NC) as a probe. MARCKS protein level was determined by Western blot using purified anti-MARCKS IgG1 (clone 2F12) as the primary detection antibody.

Transient Transfection—The phosphorylation site domain (PSD) of MARCKS contains the PKC-dependent phosphorylation sites and the actin filament-binding site (16). To construct a PSD-deleted MARCKS cDNA, two fragments flanking the PSD sequence (coding for 25 amino acids) were generated by polymerase chain reaction and then ligated through the *Xho*I site that was attached to the 5′-ends of oligonucleotide primers designed for the polymerase chain reaction. The resultant mutant cDNA and the wild-type MARCKS cDNA were each inserted into a mammalian expression vector pcDNA4/TO (Invitrogen, Carlsbad, CA). Isolated recombinant constructs were confirmed by restriction digests and DNA sequencing.

HBE1 is a papilloma virus-transformed human bronchial epithelial cell line (17) capable of mucin secretion when cultured in air/liquid interface. Transfection of HBE1 cells was carried out using the Effectene transfection reagent (Qiagen, Valencia, CA) according to the manufacturer's instructions. Briefly, differentiated HBE1 cells grown in air/liquid interface were dissociated by trypsin/EDTA and re-seeded in 12-well culture plates at 1×10^5 cells/cm^2. After overnight incubation, cells were transfected with the wild-type MARCKS cDNA, the PSD-truncated MARCKS cDNA, or vector DNA. Cells were cultured for 48 h to allow gene expression and then exposed to secretagogues and mucin secretion measured by ELISA. All transfections were carried out in the presence of pcDNA4/TO/*lacZ* plasmid (Invitrogen) (DNA ratio 6:1, total 1 μg DNA, ratio of DNA to Effectene reagent = 1:25) to monitor variations in transfection efficiency. Results showed no significant difference in β-galactosidase activities in cell lysates isolated from the transfected cells, indicating similar transfection efficiency among different DNA constructs (data not shown).

Protein Phosphatase Activity Assay—PP1 and PP2A activities were measured using a protein phosphatase assay system (Life Technologies, Inc.) as described (18) with modification. Briefly, NHBE cells were treated with 8-Br-cGMP or medium alone for 5 min. Cells were then scraped into a lysis buffer (50 mM Tris-HCl (pH 7.4), 0.1% β-mecaptoethanol, 0.1 mM EDTA, 1 mM benzamidine, 10 μg/ml pepstatin A, 10 μg/ml leupeptin) and disrupted by sonication for 20 s at 4 °C. Cell lysates were centrifuged and the supernatants saved for phosphatase activity assay. The assay was performed using ^{32}P-labeled phosphorylase A as a substrate. Released $^{32}P_i$ was counted by scintillation. The protein concentration of each sample was determined by the Bradford assay. PP2A activity was expressed as the sample total phosphatase activity minus the activity remaining in the presence of 1 nM okadaic acid. PP1 activity was expressed as the difference between the activities remaining in the presence of 1 nM and 1 μM okadaic acid, respectively.

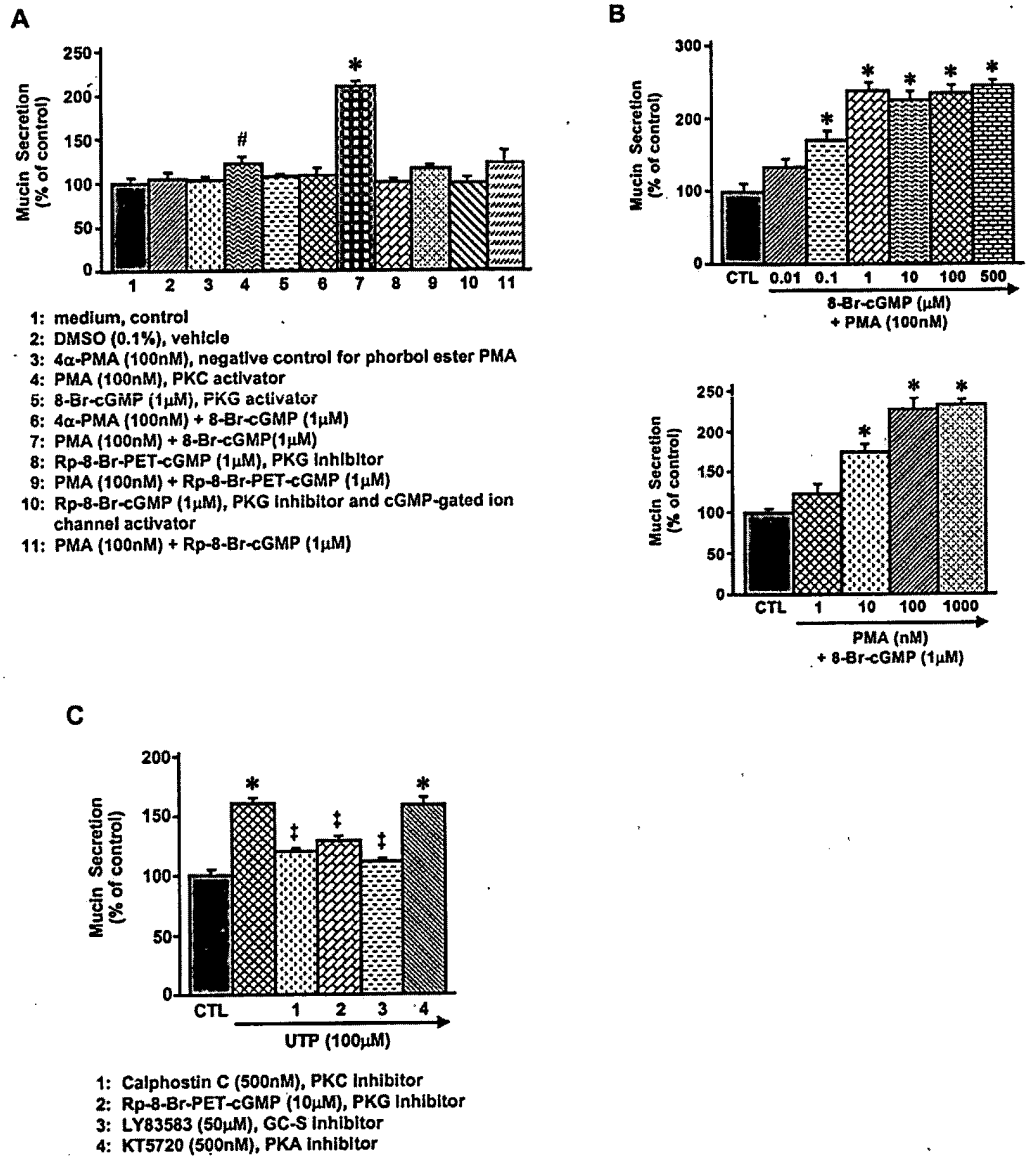


FIG. 1. **Mucin hypersecretion by NHBE cells is maximized by activation of both PKC and PKG.** *A* and *B*, NHBE cells were exposed to indicated reagent(s) or medium alone (*CTL*) for 15 min. *C*, NHBE cells were preincubated with the indicated inhibitor for 15 min and then stimulated with 100 μM UTP for 2 h. Secreted mucin in response to the treatment was collected and assayed by ELISA. Data are presented as mean ± S.E. ($n = 6$ at each point). *, significantly different from medium control ($p < 0.05$). #, different from medium control ($0.05 < p < 0.1$). ‡, significantly different from UTP stimulation ($p < 0.05$).

Protein phosphatase activities were reported as nmol of P_i released per min/mg total protein.

Cytotoxicity Assay—All reagents used in treating NHBE cells were examined for cytotoxicity by measuring the total release of lactate dehydrogenase from the cells. The assay was carried out using the Promega Cytotox 96 Kit according to the manufacturer's instructions. All experiments were performed with reagents at non-cytotoxic concentrations.

Statistical Analysis—Data were analyzed for significance using one-way analysis of variance with Bonferroni post-test corrections. Differences between treatments were considered significant at $p < 0.05$.

RESULTS

Mucin Hypersecretion from NHBE Cells Involves Activation of Both PKC and PKG

To determine the potential role of PKC and/or PKG in the mucin secretory process, NHBE cells were exposed to the following two specific protein kinase activators: the phorbol ester, phorbol 12-myristate 13-acetate (PMA), for activation of PKC, and the nonhydrolyzable cGMP analogue, 8-Br-cGMP, for activation of PKG. Preliminary studies examining mucin secretion in response to PMA stimulation at various concentrations for different times (up to 1 μM for 2 h) indicated that activation of PKC alone did not induce significant mucin secretion from NHBE cells, although a moderate secretory response was repeatedly observed at PMA concentrations higher than 100 nM ($0.05 < p < 0.1$). Also, the cells did not respond to the cGMP analogues at concentrations as high as 500 μM for up to 2 h of exposure. However, a combination of PMA + 8-Br-cGMP, affecting dual activation of PKC and PKG, provoked a rapid increase in secretion, approximately doubling it within 15 min of exposure (Fig. 1*A*). This secretory response induced by PMA + 8-Br-cGMP was concentration-dependent, with maximal stimulation at 100 nM PMA + 1 μM 8-Br-cGMP (Fig. 1*B*).

UTP is a well defined pathophysiologically relevant mucin secretagogue (19). Our preliminary studies showed that UTP (100 μM) could induce a significant increase in mucin secretion from NHBE cells after a 2-h exposure. To determine whether PKC and PKG were involved in regulation of mucin secretion in response to a pathophysiological stimulus, effects of PKC/PKG

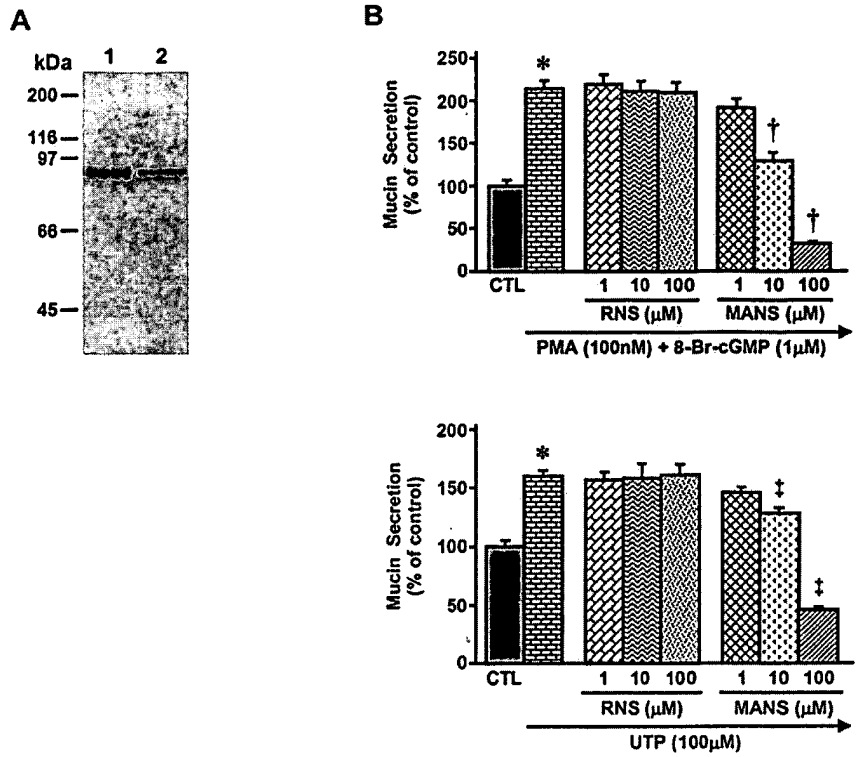


FIG. 2. **MARCKS protein is a key component of the mucin secretory pathway.** *A*, MARCKS is myristoylated and mostly membrane-associated in NHBE cells. Cells were labeled with [^{3}H]myristic acid overnight, and the membrane (*lane 1*) and the cytosol (*lane 2*) fractions were then isolated by differential centrifugation. MARCKS protein in each fraction was examined by immunoprecipitation as described. *B*, the MANS peptide blocks mucin hypersecretion induced by PMA + 8-Br-cGMP or UTP in a concentration-dependent manner. NHBE cells were preincubated with the indicated peptide for 15 min and then exposed to PMA (100 nM) + 8-Br-cGMP (1 μM) for 15 min or UTP (100 μM) for 2 h. Mucin secretion was measured by ELISA. Data are presented as mean ± S.E. (n = 6 at each point). *, significantly different from medium control ($p < 0.05$); †, significantly different from PMA + 8-Br-cGMP stimulation ($p < 0.05$); ‡, significantly different from UTP stimulation ($p < 0.05$).

inhibitors on UTP-induced mucin secretion were investigated. NHBE cells were preincubated with various inhibitors for 15 min and then exposed to UTP (100 μM) plus the inhibitor for 2 h, and secreted mucin was measured by ELISA. The results indicated that mucin secretion provoked by UTP similarly required both PKC and PKG activities, as the secretory response was attenuated independently by the PKC inhibitor calphostin C (500 nM), the PKG inhibitor R_p-8-Br-PET-cGMP (10 μM), or the soluble guanylyl cyclase (GC-S) inhibitor LY83583 (50 μM) but not by the protein kinase A (PKA) inhibitor KT5720 (500 nM) (Fig. 1*C*). Apparently, mucin secretion in NHBE cells is regulated by a signaling mechanism involving both PKC and PKG.

MARCKS Is a Key Molecule Linking PKC/PKG Activation to Mucin Secretion in NHBE Cells

To address the signaling mechanism downstream of protein kinase activation, we turned our attention to a specific cellular substrate of PKC, MARCKS protein, that might play a role in linking kinase activation to granule release. We first confirmed the presence of MARCKS in NHBE cells by [^{3}H]myristic acid-labeled immunoprecipitation assay. As illustrated in Fig. 2*A*, MARCKS was expressed in NHBE cells, and the majority of this protein was membrane-associated under unstimulated conditions. Then a role for MARCKS as a key regulatory component of the mucin secretory pathway was demonstrated in three different ways.

Peptide Blocking Studies—NHBE cells were preincubated with either the MANS or the RNS peptide (1–100 μM) for 15 min, and then PMA (100 nM) + 8-Br-cGMP (1 μM) or UTP (100 μM) was added, and cells were incubated for an additional 15 min or 2 h, respectively. Mucin secretion was measured by ELISA. As shown in Fig. 2*B*, incubation of NHBE cells with the MANS peptide resulted in a concentration-dependent suppression of mucin secretion in response to PKC/PKG activation or UTP stimulation, whereas the control peptide (RNS) did not affect secretion at these same concentrations. Effects of the MANS peptide were not related to cytotoxicity or general repression of cellular metabolic activity, as neither the MANS nor the RNS peptide affected lactate dehydrogenase release or [^{3}H]deoxyglucose uptake by the cells (data not shown).

Antisense Oligonucleotide Studies—To demonstrate further MARCKS as a key signaling component of the mucin secretory pathway, the effect of an antisense oligonucleotide directed against MARCKS on mucin secretion was examined. As illustrated in Fig. 3, this antisense oligonucleotide down-regulated both mRNA and protein levels of MARCKS in NHBE cells and significantly attenuated mucin secretion induced by PMA + 8-Br-cGMP, whereas a control oligonucleotide had no effect.

Transient Transfection of HBE1 Cells—Transfection of HBE1 cells with the PSD-truncated MARCKS cDNA resulted in significant inhibition of mucin secretion in response to PMA + 8-Br-cGMP stimulation, whereas transfection with the wild-type MARCKS cDNA or the vector DNA had no effect (Fig. 4).

MARCKS Serves as a Convergent Signaling Molecule Mediating Cross-talk of PKC and PKG Pathways

To reveal molecular events by which MARCKS links kinase activation to mucin secretion, phosphorylation of MARCKS in response to PKC/PKG activation was investigated in depth. As illustrated in Fig. 5*A*, PMA (100 nM) induced a significant increase (3–4-fold) in MARCKS phosphorylation in NHBE cells, and this phosphorylation was attenuated by the PKC inhibitor calphostin C (500 nM). Once phosphorylated, MARCKS was

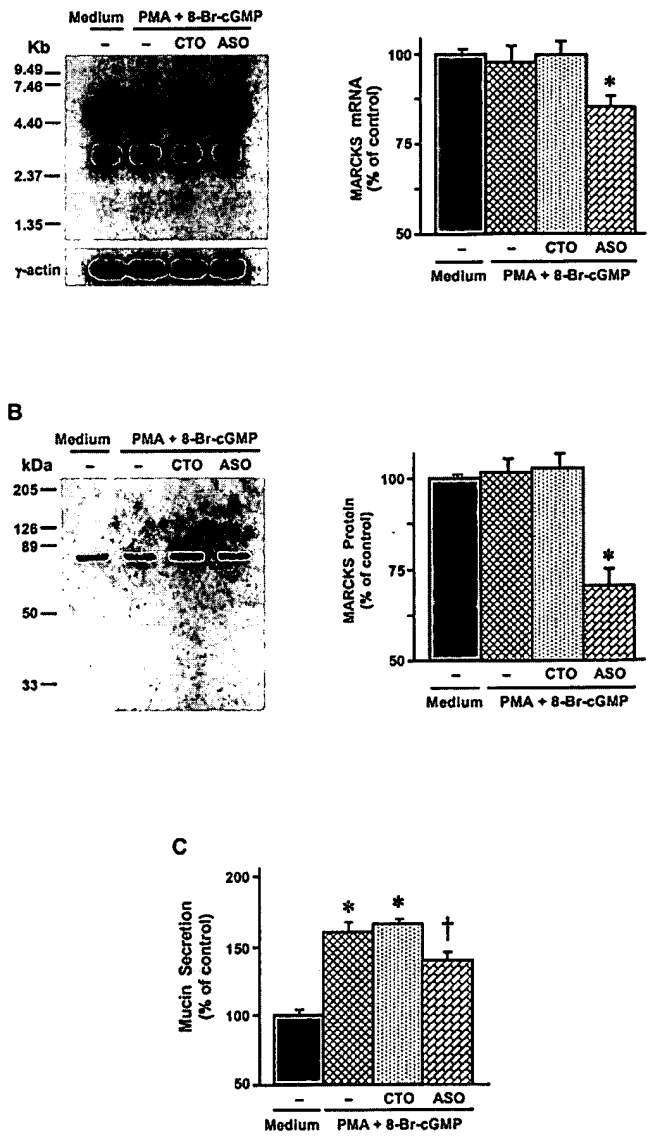
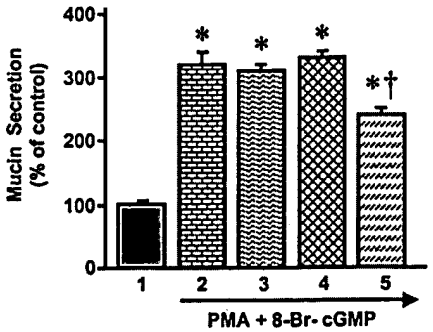


FIG. 3. **Antisense oligonucleotide directed against MARCKS down-regulates MARCKS expression and attenuates mucin hypersecretion.** NHBE cells were treated with the antisense or the control oligonucleotide for 3 days and then stimulated with PMA (100 nM) + 8-Br-cGMP (1 μM) for 15 min. Mucin secretion was analyzed by ELISA. Total RNA and protein were isolated from treated cells. MARCKS mRNA was assessed by Northern hybridization, and protein was assessed by Western blot. *A,* Northern blot showed a decrease of ~15% in MARCKS mRNA compared with controls. *B,* Western blot showed a decrease of ~30% in MARCKS protein. *C,* mucin hypersecretion was attenuated significantly by the antisense oligonucleotide, whereas the control oligonucleotide had no effect. Data are presented as mean ± S.E. (n = 6 at each point). *, significantly different from medium control ($p < 0.05$); †, significantly different from PMA + 8-Br-cGMP stimulation ($p < 0.05$). *CTO,* control oligonucleotide; *ASO,* antisense oligonucleotide.

translocated from the plasma membrane to the cytoplasm (Fig. 5*B*). Activation of PKG by 8-Br-cGMP (1 μM), another kinase activation event necessary for provoking mucin secretion, did not lead to MARCKS phosphorylation, but, in fact, the opposite effect was observed: MARCKS phosphorylation induced by PMA was reversed by 8-Br-cGMP (Fig. 6*A*). This effect of 8-Br-cGMP was not due to suppression of PKC activity, as the PMA-induced phosphorylation could be reversed by subsequent addition of 8-Br-cGMP to the cells (Fig. 6*B*). Therefore, PKG activation clearly resulted in dephosphorylation of MARCKS.



1 & 2: Control (no DNA)
3: Vector, pcDNA4/TO
4: Wild-type MARCKS construct
5: PSD-deleted MARCKS mutant construct

FIG. 4. **Transfection of PSD-deleted mutant MARCKS results in repression of mucin hypersecretion.** HBE1 cells were transiently transfected with wild-type MARCKS, PSD-deleted MARCKS, or vector DNA as described under "Experimental Procedures." After 48 h, cells were exposed to PMA (100 nM) + 8-Br-cGMP (1 μM) (*lanes 2–5*) or medium alone (*lane 1*) for 15 min. Mucin secretion was analyzed by ELISA. Data are presented as mean ± S.E. (n = 6 at each point). *, significantly different from medium control ($p < 0.05$); †, significantly different from PMA + 8-Br-cGMP stimulation ($p < 0.05$).

Further investigation showed that PKG-induced MARCKS dephosphorylation was blocked by 500 nM okadaic acid, a protein phosphatase (type 1 and/or 2A (PP1/2A)) inhibitor (Fig. 6*A*, *lane 6*). Thus, it appeared that the dephosphorylation was mediated by PP1 and/or PP2A. To define the subtype of protein phosphatase involved, a novel and more specific inhibitor of PP2A, fostriecin (IC_{50} = 3.2 nM) (20), was utilized in additional phosphorylation studies. As illustrated in Fig. 6*C*, fostriecin inhibited PKG-induced MARCKS dephosphorylation in a concentration-dependent manner (1–500 nM), suggesting that PKG induced the dephosphorylation via activation of PP2A. To confirm further activation of PP2A by PKG in NHBE cells, cytosolic PP1 and PP2A activities were determined after exposure of the cells to 8-Br-cGMP. PP2A activity was increased approximately 3-fold (from 0.1 to 0.3 nmol/min/mg proteins, $p < 0.01$) at concentrations of 8-Br-cGMP as low as 0.1 μM, whereas PP1 activity remained unchanged. These data clearly indicate that PP2A is activated by PKG and is responsible for the dephosphorylation of MARCKS. Accordingly, this PP2A activity appeared critical for mucin secretion to occur; when PKG-induced MARCKS dephosphorylation was blocked by okadaic acid or fostriecin, the secretory response to PKC/PKG activation or UTP stimulation was ameliorated (Fig. 7).

MARCKS Associates with Actin and Myosin in the Cytoplasm

Radiolabeled immunoprecipitation assay revealed that MARCKS associated with two other proteins (~200 and ~40 kDa) in the cytoplasm (Fig. 8). Matrix-assisted laser desorption ionization/time of flight mass spectrometry/internal sequence analysis indicated that these two MARCKS-associated proteins were myosin (heavy chain, non-muscle type A) and actin, respectively.

DISCUSSION

Transformed cell lines of airway epithelium tend to contain altered signaling pathways, and cell lines or nondifferentiated cells may not respond to exogenous stimuli in a manner similar to differentiated cells *in vivo*. The NHBE cells utilized in the present study were cultured at the air/liquid interface, resulting in fully differentiated primary cell cultures that maintained a well documented structure and function similar to the

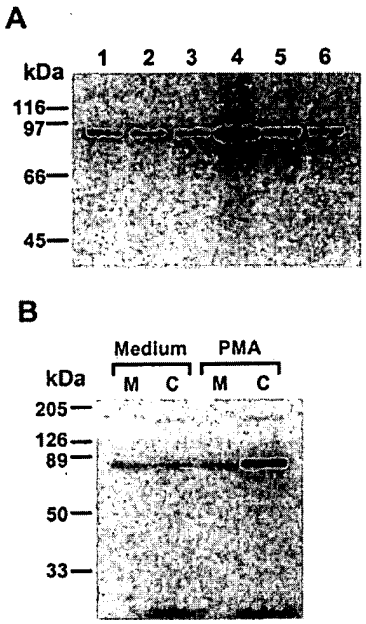
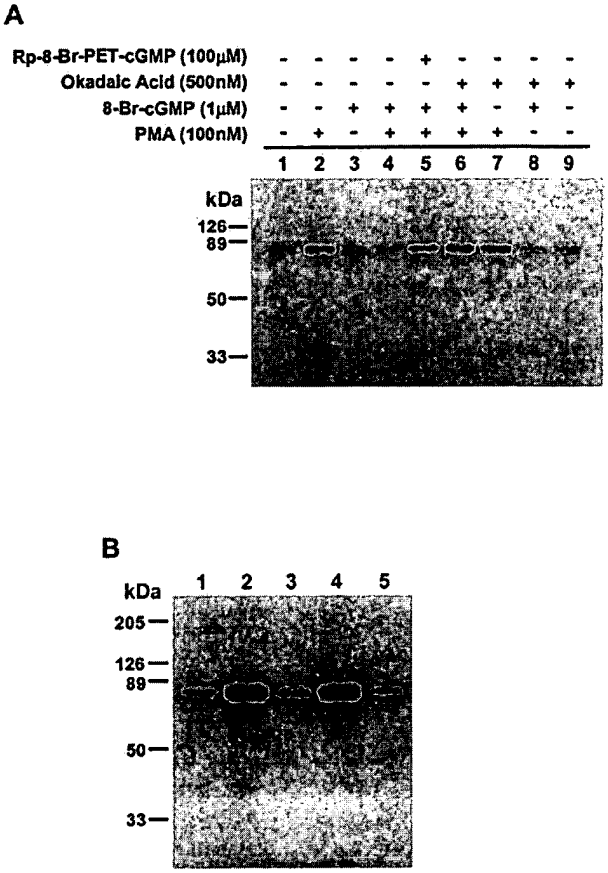


FIG. 5. **PKC-dependent phosphorylation releases MARCKS from the plasma membrane to the cytoplasm.** *A,* activation of PKC results in MARCKS phosphorylation in NHBE cells. Cells were labeled with [^{32}P]orthophosphate for 2 h and then exposed to the stimulatory and/or inhibitory reagents. MARCKS phosphorylation in response to the treatments was evaluated by immunoprecipitation as described. *Lane 1,* medium control; *lane 2,* the vehicle, 0.1% Me_2SO; *lane 3,* 100 nM 4α-PMA; *lane 4,* 100 nM PMA; *lane 5,* 100 nM PMA + 500 nM calphostin C; *lane 6,* 500 nM calphostin C. *B,* phosphorylated MARCKS is translocated from the plasma membrane to the cytoplasm. ^{32}P-Labeled cells were exposed to PMA (100 nM) or medium alone for 5 min, and then the membrane and the cytosol fractions were isolated. MARCKS phosphorylation in each fraction was evaluated by immunoprecipitation assay. *M,* membrane fraction; *C,* cytosolic fraction.



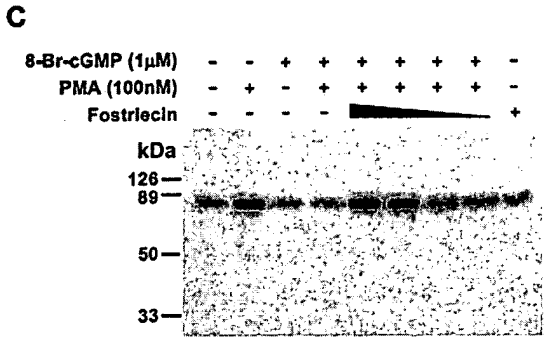


FIG. 6. **PKG induces dephosphorylation of MARCKS by activating PP2A.** NHBE cells were labeled with [^{32}P]orthophosphate and then exposed to the indicated reagents. MARCKS phosphorylation in response to the treatments was evaluated by immunoprecipitation assay. *A,* 8-Br-cGMP reversed MARCKS phosphorylation induced by PMA, and this effect of 8-Br-cGMP could be blocked by R_p-8-Br-PET-cGMP (PKG inhibitor) or okadaic acid (PP1/2A inhibitor). *B,* PMA-induced phosphorylation of MARCKS was reversed by subsequent exposure of cells to 8-Br-cGMP. *Lane 1,* medium alone for 8 min; *lane 2,* 100 nM PMA for 3 min; *lane 3,* 100 nM PMA for 3 min and then with 1 μM 8-Br-cGMP for 5 min; *lane 4,* 100 nM PMA for 8 min; *lane 5,* medium alone for 3 min and then 100 nM PMA + 1 μM 8-Br-cGMP for 5 min. *C,* 8-Br-cGMP-induced MARCKS dephosphorylation was attenuated by fostriecin in a concentration-dependent manner. ◣ from *left* to *right*: 500, 100, 10, and 1 nM fostriecin; the *last lane*: 500 nM fostriecin alone.

in vivo one (8, 21–23). This air/liquid methodology to culture airway epithelial cells was developed several years ago to provide an ideal *in vitro* model system to study mechanisms involved in various cellular processes in airway epithelium. The cell cultures contain secretory cells as well as ciliated and basal cells. Responses of these cells relate to the *in vivo* situation as they maintain the heterogeneous cell-cell contacts and polarized epithelial structure that no doubt influence their behavior *in situ*. Although MARCKS is likely present in non-secretory cells also, the clear and rapid causal associations between modifications of MARCKS and secretory outcomes suggest that mucin secretion is the direct effect of the MARCKS-related molecular events, as demonstrated in this study, occurring within the secretory cells.

Previous studies with transformed cell lines and airway epithelial cells isolated from various animal species have suggested involvement of PKC or PKG in mucin secretion (3–7). We report here for the first time that concurrent activation of both PKC and PKG maximized mucin secretion from differentiated NHBE cells, and activation of either kinase alone is not sufficient to elicit a robust secretory response. These results are in general agreement with most of the previous findings. We did document an enhanced secretory response to PMA alone (Fig. 1, *column 4*), although the magnitude of the response was much less than that observed by others (4) in a rat goblet-like cell line. In addition, although we have reported previously that a cGMP analogue could induce significant mucin secretion from cultured guinea pig tracheal epithelial cells (7), it should be noted that this response did not reach significant levels until 8 h of exposure. A secretory response with such a long lag period is unlikely to be a direct effect and probably involves *de novo* protein synthesis as opposed to release of preformed and stored cytoplasmic granules. Nevertheless, the apparent synergistic effect involving cooperative activation of both PKC and PKG suggests a complex and stringent signaling mechanism mediating mucin secretion in differentiated human airway epithelium.

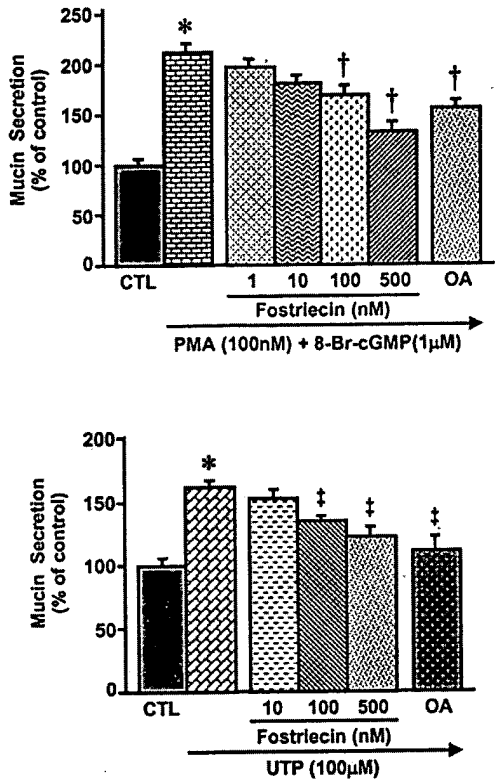


FIG. 7. **PP2A is an essential component of the mucin secretory pathway.** NHBE cells were preincubated with the indicated concentration of fostriecin, okadaic acid (500 nM), or medium alone for 15 min and then stimulated with PMA (100 nM) + 8-Br-cGMP (1 μM) for 15 min or with UTP (100 μM) for 2 h. Secreted mucin was measured by ELISA. Data are presented as mean ± S.E. ($n = 6$ at each point). *, significantly different from medium control ($p < 0.05$); †, significantly different from PMA + 8-Br-cGMP stimulation ($p < 0.05$); ‡, significantly different from UTP stimulation ($p < 0.05$).

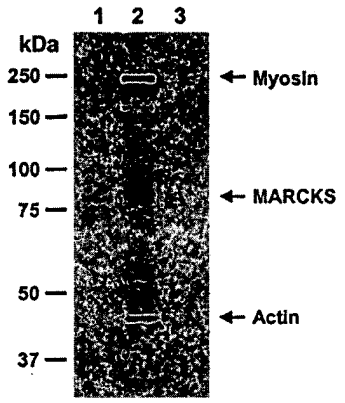


FIG. 8. **MARCKS associates with actin and myosin in the cytoplasm.** NHBE cells were labeled with [^{3}H]leucine and [^{3}H]proline overnight, and the membrane and the cytosol fractions were prepared as described under "Experimental Procedures." Isolated fractions were precleared with the nonimmune control antibody (6F6). The cytosol was then divided equally into two fractions and used for immunoprecipitation carried out in the presence of 10 μM cytochalasin D (Biomol, Plymouth Meeting, PA) with the anti-MARCKS antibody 2F12 (*lane 2*) and the nonimmune control antibody 6F6 (*lane 3*), respectively. MARCKS protein in the membrane fraction was also assessed by immunoprecipitation using the antibody 2F12 (*lane 1*). The precipitated protein complex was resolved by 8% SDS-polyacrylamide gel electrophoresis and visualized by enhanced autoradiography. MARCKS appeared to associate with two cytoplasmic proteins with molecular masses of ~200 and ~40 kDa, respectively. These two MARCKS-associated proteins were excised from the gel and analyzed by matrix-assisted laser desorption ionization/time of flight mass spectrometry/internal sequencing (the Protein/DNA Technology Center of Rockefeller University, New York). The obtained peptide mass and sequence data were used to search protein data bases via Internet programs ProFound and MS-Fit. Results indicate that they are myosin (heavy chain, nonmuscle type A) and actin, respectively.

To address involvement of PKG in the secretory process, 8-Br-cGMP was utilized in these studies. Although the primary physiological effect of 8-Br-cGMP is to activate PKG (24), it also has been reported to act as an agonist for cGMP-gated ion channels in some cells (25) and, at high concentrations, to cross-activate PKA (reviewed in Ref. 26). To preclude the possibility that cGMP-gated ion channels and/or PKA may play a role in mucin secretion by NHBE cells, R_p-8-Br-cGMP, a unique cGMP analogue that can activate cGMP-gated ion channels similar to 8-Br-cGMP but inhibit PKG activity (25), was used as an agonist to distinguish the effects of PKG and cGMP-gated ion channels on mucin release. As illustrated in Fig. 1*A* (*column 11*), R_p-8-Br-cGMP did not enhance mucin secretion when added to the cells with PMA. Likewise, the specific PKA inhibitor, KT5720 (500 nM), did not affect mucin secretion induced by either PMA + 8-Br-cGMP (data not shown) or UTP (Fig. 1*C*, *column 4*). These studies negate the possibility that cGMP-gated ion channels or PKA are associated with mucin secretion, indicating that activation of PKG in NHBE cells is the mechanism whereby 8-Br-cGMP contributes to enhanced secretion. Furthermore, because UTP-induced mucin hypersecretion can be attenuated by the soluble guanylyl cyclase (GC-S) inhibitor LY83583, it is likely that activation of PKG occurs via the signaling pathway of nitric oxide (NO) → GC-S → cGMP → PKG, as illustrated previously (7) in differentiated guinea pig tracheal epithelial cells *in vitro*.

Given the participation of both PKC and PKG in the mucin secretory process, we began to examine potential intracellular substrates of these enzymes that could play a role in signaling events downstream of the kinase activation. Numerous intracellular substrates can be phosphorylated by PKC or PKG, and phosphorylation by PKC of one such substrate, MARCKS protein, seemed to be of particular interest. The specific biological function of MARCKS is not clear; however, MARCKS phosphorylation has been observed to correlate with a number of cellular processes involving PKC signaling and cytoskeletal contraction, such as cell movement, mitogenesis, and neural transmitter release (reviewed in Ref. 27). Because the dynamic process of secretion requires both kinase activation and translocation of intracellular granules to the cell periphery, MARCKS appeared to be an ideal candidate for a mediator molecule connecting PKC/PKG activation and mucin granule exocytosis.

Direct involvement of MARCKS in mucin secretion by NHBE cells was demonstrated by three separate lines of evidence. First, mucin secretion in response to stimulation by PMA + 8-Br-cGMP or UTP was inhibited in a concentration-dependent manner by the MANS peptide, which had the amino acid sequence identical to the N-terminal region of MARCKS, whereas the corresponding control peptide (RNS), containing the same amino acid composition but arranged in random order, did not affect secretion. The N-terminal myristoylated domain of MARCKS is known to mediate the MARCKS-membrane association. As indicated in the hypothetical mechanism illustrated in Fig. 9, MARCKS may function as a molecular linker by interacting with granule membranes at its N-terminal domain and binding to actin filaments at its PSD site, thereby tethering granules to the contractile cytoskeleton for movement and exocytosis. It is also conceivable that attachment of MARCKS to the granules after it is released into the cytoplasm may be guided by specific targeting proteins or some other forms of protein-protein interactions in which the N-terminal domain of MARCKS is involved. In either case, the MANS peptide would

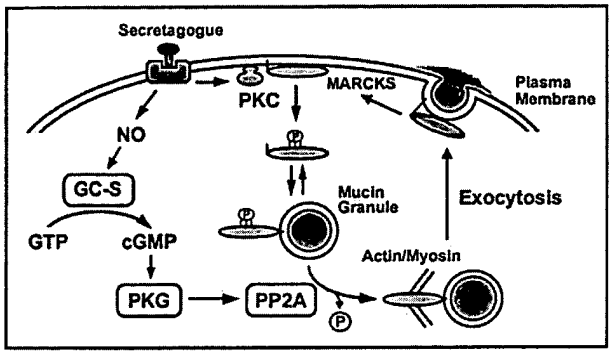


FIG. 9. **Hypothetical signaling mechanism controlling mucin secretion by human airway epithelial cells.** Mucin secretagogue interacts with airway epithelial (goblet) cells and activates two separate protein kinases, PKC and PKG. Activated PKC phosphorylates MARCKS, causing MARCKS translocation from the plasma membrane to the cytoplasm, whereas PKG, activated via the nitric oxide (NO) → GC-S → cGMP → PKG pathway, in turn activates a cytoplasmic PP2A, which dephosphorylates MARCKS. This dephosphorylation stabilizes MARCKS attachment to the granule membranes. In addition, MARCKS also interacts with actin and myosin, thereby linking granules to the cellular contractile machinery for subsequent movement and exocytotic release.

act to inhibit competitively targeting of MARCKS to the membranes of mucin granules, thereby blocking secretion.

The second line of evidence was provided by the inhibitory effect of a MARCKS-specific antisense oligonucleotide on mucin secretion. As shown in Fig. 3, the antisense oligonucleotide down-regulated MARCKS mRNA and protein levels in NHBE cells and substantially attenuated mucin secretion induced by PKC/PKG activation. The inhibition was not as dramatic as that seen with the MANS peptide, which might be due to the high levels of endogenous MARCKS protein in NHBE cells and the relatively long half-life of MARCKS mRNA ($t_{1/2}$ = 4–6 h) (28).

The final line of evidence was that transfection of HBE1 cells with a PSD-deleted mutant MARCKS resulted in significant repression of mucin secretion induced by PKC/PKG activation. Deletion of the PSD would abolish the ability of MARCKS to bind to actin. As indicated in Fig. 9, by competing with native MARCKS for binding to granule membrane, the PSD-truncated MARCKS could thereby inhibit granule release as it is unable to interact with the actin filaments. One piece of data that should be addressed here is the fact that transfection of these cells with the wild-type MARCKS cDNA did not further enhance mucin secretion. Western blot assay showed that the expression level of endogenous MARCKS in HBE1 cells was quite high, comparable with that in NHBE cells, and transfection of wild-type MARCKS cDNA did not lead to notable increases in overall MARCKS protein level in these cells (data not shown). This may explain why transfection with wild-type MARCKS did not further augment secretion and also why transfection with the PSD-deleted MARCKS only partially hindered mucin secretion.

Collectively, the above results demonstrated that MARCKS was involved integrally in the mucin secretory process. The next question to address was how MARCKS acts as a key regulatory molecule upon which PKC and PKG converge to regulate mucin secretion. A major part of the answer to this question could reside in the dynamics of MARCKS phosphorylation/dephosphorylation. As illustrated in Fig. 5, MARCKS was phosphorylated by PKC and consequently translocated from the membrane to the cytoplasm. Here, PKG appeared to induce dephosphorylation of MARCKS (Fig. 6*A*, *lane 4*, and Fig. 6*B*). This dephosphorylation was reversed by the PKG inhibitor R_p-8-Br-PET-cGMP (Fig. 6*A*, *lane 5*), indicating the dephosphorylation was specifically PKG-dependent.

How does PKG act to dephosphorylate MARCKS? An obvious mechanism is via activation of a protein phosphatase. As illustrated in Fig. 6*A* (*lane 6*), okadaic acid at 500 nM, a concentration that could inhibit both PP1 and PP2A, blocked PKG-induced dephosphorylation of MARCKS, suggesting that PKG caused dephosphorylation by activating PP1 and/or PP2A. Further studies with fostriecin and direct assay of phosphatase activities indicated that only PP2A was activated by PKG and was responsible for removal of the phosphate groups from MARCKS (Fig. 6*C*). Finally, to bring these results to a logical conclusion, either okadaic acid or fostriecin, at concentrations that inhibited PKG-induced dephosphorylation of MARCKS, attenuated mucin secretion induced by PMA + 8-Br-cGMP or UTP (Fig. 7). Thus, dephosphorylation of MARCKS by a PKG-activated PP2A appears to be an essential component of the signaling pathway leading to mucin granule exocytosis.

Results of these studies not only suggest a new paradigm for the signaling mechanism controlling exocytotic secretion of airway mucin granules but also provide the first direct evidence demonstrating a specific biological function of MARCKS in a physiological process; MARCKS serves as a key mediator molecule regulating mucin granule release in human airway epithelial cells. A hypothetical signaling pathway involving this secretory process is illustrated in Fig. 9. Specifically, elicitation of airway mucin secretion requires dual activation and synergistic actions of PKC and PKG. Activated PKC phosphorylates MARCKS, resulting in translocation of MARCKS from the inner face of the plasma membrane into the cytoplasm. Activation of PKG in turn activates PP2A, which dephosphorylates MARCKS in the cytoplasm. Because the membrane association ability of MARCKS is dependent on its phosphorylation state (9–11), this dephosphorylation would allow MARCKS to regain its membrane-binding capability and would enable MARCKS to attach to membranes of cytoplasmic mucin granules. By also interacting with actin and myosin in the cytoplasm (Fig. 8), MARCKS could then tether granules to the cellular contractile apparatus, mediating granule movement to the cell periphery and subsequent exocytotic release.

Certainly, the mechanism described above fits well into our current knowledge about mucin secretion and secretagogue activity. PKC and/or the NO → GC-S → PKG pathways have been thought to be involved in airway mucin secretion provoked by a wide range of stimuli. On a more etiological level, it also seems rational that a single intracellular molecule regulates the actual secretory event, that is movement and exocytosis of mucin granules. The need for activation of two separate protein kinases, which control phosphorylation and dephosphorylation of this single regulatory molecule, respectively, reflects another level of fine control of the secretory process. The wide distribution of MARCKS suggests the possibility that this or a similar mechanism may regulate secretion of membrane-bound granules in various cell types under normal or pathological conditions.

Acknowledgments—We thank Dr. Perry Blackshear from the NIEHS, Research Triangle Park, NC, for providing a number of MARCKS-related reagents and helpful advice.

REFERENCES

1. Adler, K. B., Fischer, B. M., Martin, L. D., and Voynow, J. A. (1998) *Res. Immunol.* **149**, 245–248
2. Cohn, L. A., and Adler, K. B. (1992) *Exp. Lung Res.* **18**, 299–322
3. Ko, K. H., Jo, M., McCracken, K., and Kim, K. C. (1997) *Am. J. Respir. Cell Mol. Biol.* **16**, 194–198
4. Abdullah, L. H., Conway, J. D., Cohn, J. A., and Davis, C. W. (1997) *Am. J. Physiol.* **273**, L201–L210
5. Abdullah, L. H., Davis, S. W., Burch, L., Yamauchi, M., Randell, S. H., Nettesheim, P., and Davis, C. W. (1996) *Biochem. J.* **316**, 943–951

6. Larivee, P., Levine, S. J., Martinez, A., Wu, T., Logun, C., and Shelhamer, J. H. (1994) *Am. J. Respir. Cell Mol. Biol.* **11,** 199–205
7. Fischer, B. M., Rochelle, L. G., Voynow, J. A., Akley, N. J., and Adler, K. B. (1999) *Am. J. Respir. Cell Mol. Biol.* **20,** 413–422
8. Krunkosky, T. M., Fischer, B. M., Martin, L. D., Jones, N., Akley, N. J., and Adler, K. B. (2000) *Am. J. Respir. Cell Mol. Biol.* **22,** 685–692
9. Seykora, J. T., Myat, M. M., Allen, L. A. H., Ravetch, J. V., and Aderem, A. (1996) *J. Biol. Chem.* **271,** 18797–18802
10. Swierczynski, S. L., and Blackshear, P. J. (1996) *J. Biol. Chem.* **271,** 23424–23430
11. McLaughlin, S., and Aderem, A. (1995) *Trends Biochem. Sci.* **20,** 272–276
12. Harlow, E., and Lane, D. (1988) *Antibodies: A Laboratory Manual,* pp. 570–573, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
13. Lin, H., Carlson, D. M., St. George, J. A., Plopper, C. G., and Wu, R. (1989) *Am. J. Respir. Cell Mol. Biol.* **1,** 41–48
14. Wright, D. T., Fischer, B. M., Li, C., Rochelle, L. G., Akley, N. J., and Adler, K. B. (1996) *Am. J. Physiol.* **271,** L854–L861
15. Spizz, G., and Blackshear, P. J. (1996) *J. Biol. Chem.* **271,** 553–562
16. Hartwig, J. H., Thelen, M., Rosen, A., Janmey, P. A., Nairn, A. C., and Aderem, A. (1992) *Nature* **356,** 618–622
17. Yankaskas, J. R., Haizlip, J. E., Conrad, M., Koval, D., Lazarowski, E., Paradiso, A. M., Rinehart, C. A., Jr., Sarkadi, B., Schlegel, R., and Boucher, R. C. (1993) *Am. J. Physiol.* **264,** C1219–C1230
18. Huang, X. C., Sumners, C., and Richards, E. M. (1996) *Adv. Exp. Med. Biol.* **396,** 209–215
19. Lethem, M. I., Dowell, M. L., Van Scott, M., Yankaskas, J. R., Egan, T., Boucher, R. C., and Davis, C. W. (1993) *Am. J. Respir. Cell Mol. Biol.* **9,** 315–322
20. Walsh, A. H., Cheng, A., and Honkanen, R. E. (1997) *FEBS Lett.* **416,** 230–234
21. Adler, K. B., Cheng, P. W., and Kim, K. C. (1990) *Am. J. Respir. Cell Mol. Biol.* **2,** 145–154
22. Kaartinen, L., Nettesheim, P., Adler, K. B., and Randell, S. H. (1993) *In Vitro Cell. Dev. Biol. Anim.* **29A,** 481–492
23. Gray, T. E., Guzman, K., Davis, C. W., Abdullah, L. H., and Nettesheim, P. (1996) *Am. J. Respir. Cell Mol. Biol.* **14,** 104–112
24. Sekhar, K. R., Hatchett, R. J., Shabb, J. B., Wolfe, L., Francis, S. H., Wells, J. N., Jastorff, B., Butt, E., Chakinala, M. M., and Corbin, J. D. (1992) *Mol. Pharmacol.* **42,** 103–108
25. Wei, J. Y., Cohen, E. D., Genieser, H. G., and Barnstable, C. J. (1998) *J. Mol. Neurosci.* **10,** 53–64
26. Schmidt, H. H., Lohmann, S. M., and Walter, U. (1993) *Biochim. Biophys. Acta* **1178,** 153–175
27. Aderem, A. (1992) *Trends Biochem. Sci.* **17,** 438–443
28. Harlan, D. M., Graff, J. M., Stumpo, D. J., Eddy, R. L., Jr., Shows, T. B., Boyle, J. M., and Blackshear, P. J. (1991) *J. Biol. Chem.* **266,** 14399–14405

Attorney Docket No. 5051-451IP PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re: Li et al. Confirmation No.: 8515
Serial No.: 09/914,020 Art Unit: 1633
Filed: December 31, 2001 Examiner: J. Epps-Ford
For: *Methods and Compositions for Altering Mucus Secretion*

Date: June 7, 2006

Mailstop Amendment
Commissioner for Patents
PO Box 1450
Alexandria, VA 22313-1450

ATTACHMENT C

Exhibit 7

Singer et al. (2004) "*A MARCKS-related peptide blocks mucus hypersecretion in a mouse model of asthma*" Nature Medicine, 10(2):193-196.

nature medicine

A MARCKS-related peptide blocks mucus hypersecretion in a mouse model of asthma

Monique Singer[1], Linda D Martin[2], B Boris Vargaftig[3], Joungjoa Park[2], Achim D Gruber[4], Yuehua Li[2] & Kenneth B Adler[2]

Mucus hypersecretion is a crucial feature of pulmonary diseases such as asthma, chronic bronchitis and cystic fibrosis. Despite much research, there is still no effective therapy for this condition. Recently, we showed that the myristoylated, alanine-rich C-kinase substrate (MARCKS) protein is required for mucus secretion by human bronchial epithelial cells in culture[1]. Having synthesized a peptide corresponding to the N-terminal domain of MARCKS, we now show that the intratracheal instillation of this peptide blocks mucus hypersecretion in a mouse model of asthma. A missense peptide with the same amino acid composition has no effect. Based on quantitative histochemical analysis of the mouse airways, the peptide seems to act by blocking mucus release from goblet cells, possibly by inhibiting the attachment of MARCKS to membranes of intracellular mucin granules. These results support a pivotal role for MARCKS protein, specifically its N-terminal region, in modulating this secretory process in mammalian airways. Intratracheal administration of this MARCKS-related peptide could therapeutically reduce mucus secretion in the airways of human patients with asthma, chronic bronchitis and cystic fibrosis.

Hypersecretion of mucin (the glycoprotein component of mucus) occurs in several respiratory diseases, including asthma, chronic bronchitis and cystic fibrosis, and is a risk factor for mortality in these patient groups. Currently, there are no therapies available to treat mucus hypersecretion. We recently showed that MARCKS, an 80- to 87-kDa protein that is a target for phosphorylation by protein kinase C, is required for mucin secretion in human bronchial epithelial cells in culture[1]. We also showed that an evolutionarily conserved, N-terminal, 24–amino acid fragment of MARCKS—myristoylated N-terminal sequence (MANS), or MARCKS-related peptide—inhibits mucin release in a concentration-dependent manner *in vitro*. In contrast, a missense control peptide with the same amino acid composition had no effect[1].

To test the effect of the MARCKS-related peptide *in vivo*, we used the ovalbumin (OVA)-sensitized mouse, a well-characterized model of allergic airway inflammation resembling human asthma[2–5]. To elicit mucin hypersecretion, we exposed OVA-sensitized mice of the BP2 strain to aerosolized methacholine for 90 s, 72 h after OVA challenge[4,5]. One group of mice was pretreated intratracheally (i.t.), 15 min before methacholine, with 50 μl MANS peptide in saline at 10, 100 or 140 μM (~1–15 mg /kg). A second group was pretreated with the missense peptide (random N-terminal sequence, or RNS) at 100 or 140 μM, and a third group was pretreated with saline alone. The amount of mucin released into tracheobronchial lavage fluid 30 min after methacholine exposure was measured by a mucin-specific ELISA, as described previously[3–5]. In mice treated with saline only, inhalation of methacholine caused an approximately fivefold increase in mucin secretion (Fig. 1a). Pretreatment with the MANS peptide resulted in a concentration-dependent decrease in this response, whereas pretreatment with the missense RNS peptide had no effect at either dose (Fig. 1a). In mice not exposed to methacholine, i.t. delivery of MANS peptide for 45 min decreased basal, constitutive levels of mucin secretion by 34 ± 7% and 69 ± 11% at 10 and 100 μM, respectively, compared with saline-treated mice. RNS peptide had no effect on basal mucin secretion. Histological examination of airways and analysis of bronchoalveolar lavage (BAL) fluid revealed no signs of injury or toxicity for up to 24 h after instillation of either peptide. FITC-conjugated MANS (100 μM) instilled i.t. into a mouse airway was detected within epithelial cells 15 min later, indicating intracellular localization.

To detect possible strain-specific effects of the peptides, we conducted similar studies in BALB/c mice. The results of these experiments were essentially the same as those observed in experiments with BP2 mice (Fig. 1b). The inhibitory effect of the MANS peptide was not restricted to a single secretory stimulus, as indicated by data showing inhibitory effects of MANS peptide on secretion induced by intraperitoneal (i.p.) delivery of 150 mg/kg pilocarpine (Fig. 1c).

To gain insight into the mode of action of MANS peptide, we examined the airways of OVA-exposed mice that had or had not received peptide treatment. Histochemical staining with periodic acid–Schiff (PAS) and hematoxylin revealed that airways exposed to OVA alone contained numerous mucin-filled goblet cells (Fig. 2a),

[1]Unité de Pharmacologie Cellulaire, Institut Pasteur, 28, rue du Dr. Roux, 75724 Paris Cedex 15, France. [2]Department of Molecular Biomedical Sciences, North Carolina State University, College of Veterinary Medicine, 4700 Hillsborough Street, Raleigh, North Carolina 27606, USA. [3]Department of Pharmacology, Institute of Biomedical Sciences, University of Sao Paulo, Sao Paulo 05508-900, Brazil. [4]Department of Pathology, School of Veterinary Medicine Hannover, Buenteweg 17, 30559 Hannover, Germany. Correspondence should be addressed to K.B.A. (Kenneth_Adler@ncsu.edu).

Published online 11 January 2004; doi:10.1038/nm983

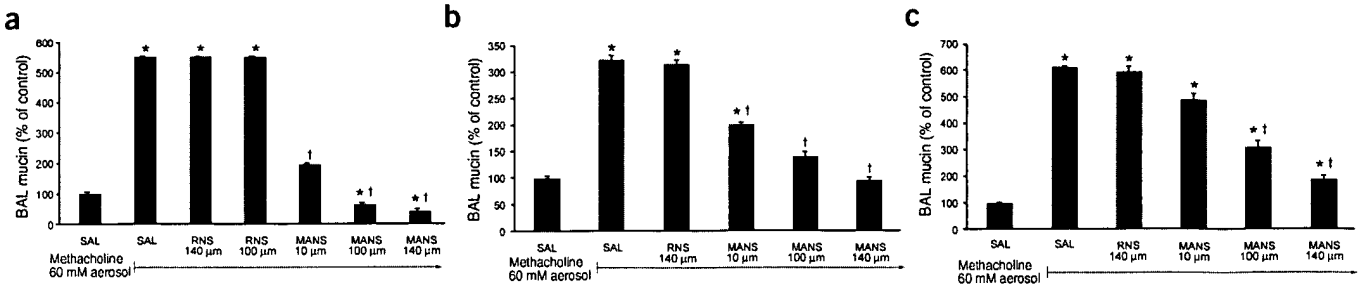


Figure 1 Effects of peptides on mucin secretion in mouse airways. (a) In BP2 mice challenged and sensitized with OVA, MANS peptide, but not missense RNS peptide, attenuates mucin secretion in response to methacholine, in a concentration-dependent manner. (b,c) In BALB/c mice sensitized and challenged with OVA, release of mucin into airways in response to inhaled aerosolized methacholine (b) or i.p. pilocarpine (c) is attenuated by pretreatment with MANS peptide, whereas RNS peptide does not affect mucin secretion. *, $P < 0.05$ compared with control; †, $P < 0.05$ compared with methacholine stimulation.

whereas upon methacholine exposure, PAS-positive material was lost from the airway epithelium and immunoreactive mucin appeared in the lavage fluid (Fig. 2b). Pretreatment with missense RNS peptide (Fig. 2c) had no effect, whereas pretreatment with MANS peptide (Fig. 2d–f) markedly reduced the effects of methacholine in a concentration-dependent manner. Quantitative morphometric analysis of these sections confirmed a concentration-dependent retention of mucin in airways of MANS-treated mice (Table 1).

The issue of how the MANS peptide interferes with mucin secretion has not yet been resolved. Studies of secretion using MARCKS knockout mice have not been done, as deleting the MARCKS gene causes CNS defects that result in death shortly after birth[6]. Work in a variety of isolated or cultured cell types however indicates that the phosphorylation state of MARCKS controls its affinity for actin filaments; it has been proposed that MARCKS-actin networks reduce the ability of secretory granules to penetrate the cortical cytoskeleton[7–12]. Supporting this, some studies have found that secretion can be attenuated by exposing cells to a peptide that blocks protein kinase C-mediated MARCKS phosphorylation[7,8].

This type of mechanism cannot however explain the results shown here, because mucin-secreting airway goblet cells lack a cortical actin ring, and because the N-terminal MANS peptide used in our studies does not interfere with MARCKS phosphorylation. Instead, the peptide used in our studies corresponds to the myristoylated N-terminal region of MARCKS, which regulates the interaction of MARCKS with membranes, and neither serves as a protein kinase C phosphorylation site nor binds or cross-links actin[13].

To account for the inhibitory effect of the MANS peptide in goblet cells, we propose a mechanism whereby MARCKS binds, at different sites, to secretory granule membranes and to the actin cytoskeleton. MARCKS would therefore serve as a physical link between the contractile cytoskeleton and mucin granules, and could have a role in guiding secretory granules to docking sites on the cell membrane. We have obtained biochemical evidence for such a mechanism in isolated human bronchial epithelial cells[1].

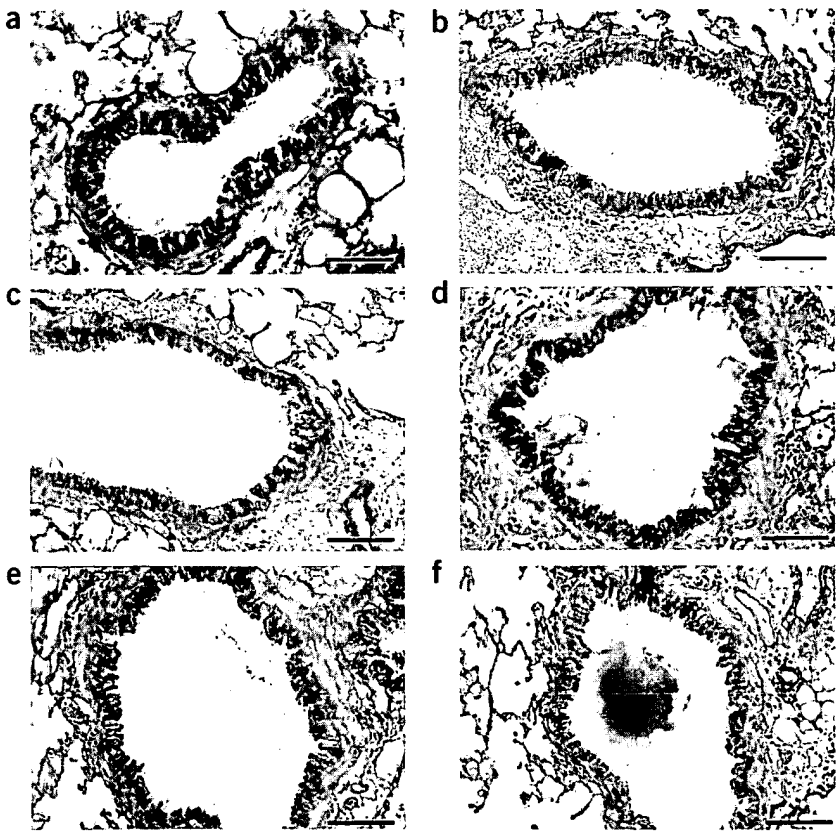


Figure 2 Representative PAS- and hematoxylin-stained sections of bronchi from sensitized and challenged BALB/c mice. (a) Mouse exposed to i.t. saline, followed by aerosolized saline. Goblet cells replete with PAS-positive intracellular mucin granules populated the epithelium. (b) Mouse exposed to i.t. saline, followed by aerosolized methacholine. Goblet cells released mucin granules; epithelium was mostly PAS-negative. (c) Mouse exposed to i.t. RNS peptide, followed by aerosolized methacholine. Epithelium was similar to that in b. (d–f) Mice exposed to i.t. MANS peptide at 140 μM (d), 100 μM (e) or 10 μM (f), followed by aerosolized methacholine. Mucin seemed to be retained in goblet cells after MANS treatment, whereas RNS peptide did not prevent methacholine-induced release of PAS-positive granules. Scale bars, 100 μm.

Here we used gold-labeled immunostaining for MARCKS to provide ultrastructural evidence of the association between MARCKS protein and membranes of mucin granules (Fig. 3a,b). MARCKS also bound membranes of mucin granules isolated from human bronchial epithelial cells; this binding was inhibited by MANS peptide (Fig. 3c). Because the MANS peptide used in these studies corresponds to the myristoylated N-terminus of MARCKS, it may be inhibiting endogenous MARCKS by competing for granule membrane binding. A sequence targeting MARCKS to specific membrane sites might reside in a region on the MARCKS protein between the myristoyl moiety and the phosphorylation-site domain[13], and might perhaps be related to the conserved multiple homology-2 (MH2) domain in this region[14].

In summary, we have shown that a peptide directed against the conserved N-terminal region of MARCKS protein inhibits mucin release *in vivo* when instilled i.t. into allergically inflamed mouse airways. The MANS peptide is the first example of a reagent with such properties. These findings lay the foundation for the development of new compounds that may find an important place in therapy for mucus hypersecretory disorders, such as those in patients with asthma, chronic bronchitis and cystic fibrosis.

METHODS

Ovalbumin-sensitized mouse model of mucus hypersecretion. All studies were approved by the Animal Care and Use Review Committee of the Institut Pasteur. Male BP2 or BALB/c mice (Centre d'Elevage R. Janvier), aged 6–8 weeks, were immunized subcutaneously with OVA (ICN Pharmaceuticals) as described previously[2]. Two weeks later, mice were anesthetized with 12% xylazine (20 mg/kg) and ketamine 500 (45 mg/kg) (Sigma), and challenged with 10 μg OVA in 50 μl of endotoxin-free 0.9% saline. OVA was administered under light anesthesia through a temporary cannula introduced into the trachea through the oral cavity.

Bronchoalveolar lavage. Mice were anesthetized i.p. with urethane (45 mg per 30 g body weight), and the trachea was incised and cannulated. BAL fluid was collected with 2.5 ml saline containing 0.005M EDTA, PMSF and DTT, all from Sigma.

Measurement of mucin secretion. Cell-free BAL fluid was assayed for secreted mucin using a previously described ELISA[4,5]. We used a mouse Muc5AC-specific antibody, raised against the peptide QTSSPNTGKTSTISTT from the mouse homolog of the *Muc5ac* gene, which recognizes lung, gastric and intestinal Muc5AC mucins[15].

Table 1 Morphometric analysis of mucin content in bronchi of BALB/c mice

	Mucin (mm^2 in airways)
Saline + MCH	0.01 ± 0.014**
OVA	1.81 ± 0.44**
OVA + MCH	0.07 ± 0.04
OVA + RNS (140 μM) + MCH	0.04 ± 0.01
OVA + MANS (140 μM) + MCH	1.44 ± 0.17**
OVA + MANS (100 μM) + MCH	0.40 ± 0.17*
OVA + MANS (10 μM) + MCH	0.14 ± 0.04

Sections from BALB/c mice, stained with PAS and hematoxylin, were analyzed for mucin content using an image analysis system. Results are mean ± s.d; $n = 25$ bronchi per group. MCH, methacholine. *, $P < 0.05$ compared with OVA + MCH; **, $P < 0.001$ compared with OVA + MCH.

Treatments. Seventy-two hours after OVA challenge, mice were instilled i.t. with a bolus of either saline, MANS peptide (identical to the first 24 amino acids of the N terminus of MARCKS; myristic acid–GAQFSKTAAKG EAAAERPGEAAVA) or missense control RNS peptide; myristic acid–GTA-PAAEGAGAEVKRASAEAKQAF), as described previously[1]. MANS or RNS peptides were administered at final concentrations of 10, 100 or 140 μM (~1–15 mg/kg). Mucin secretion was triggered 15 min later by administration of either methacholine, using a Buxco system nebulizer delivering aerosolized methacholine at 60 mM for 90 s, or i.p. injection of pilocarpine (150 mg/kg) for an additional 30 min before BAL and mucin measurement.

Histochemistry and quantitative morphometry. After the above exposures, lungs from selected BALB/c mice were flushed to remove blood, then inflated with OCT medium (Sakura Finetek) half-diluted in saline. Lungs were immersed in 10% phosphate-buffered formalin overnight at 4 °C, then embedded in paraffin. Five-micron-thick sections were stained with PAS and hematoxylin, and mucin retained in the epithelium in longitudinal sections of main-stem bronchi was quantified as described[4,16] using an image analysis system (Grastek Optilab software, version 2.1). We used five mice per treatment. For each mouse, we calculated the sum of the values of five fields per slide, for five slides, and converted the area from pixels to mm^2. All data were obtained in a blinded fashion at a magnification of ×200.

Ultrastructural immunohistochemistry. Trachea from BALB/c mice were immersion-fixed in 4% formaldehyde and 0.01% glutaraldehyde for 1h.

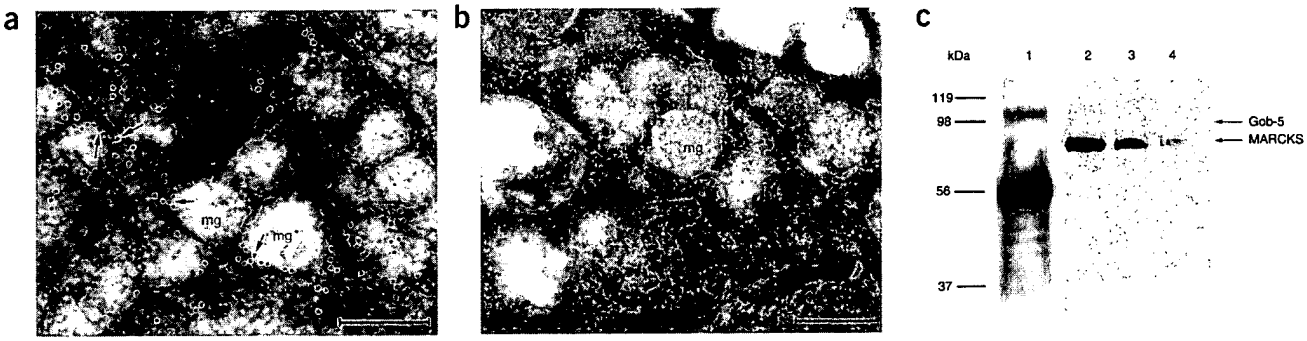


Figure 3 The association of MARCKS with mucin granule membranes is inhibited by MANS peptide. (**a**) Immunogold EM analysis of a goblet cell from mouse airway shows association of MARCKS with membranes (arrows) of mucin granules (mg). Scale bars, 300 nm. (**b**) Control for **a**, using gold-labeled preimmune IgG. (**c**) Immunoprecipitation of isolated mucin granule membranes from NHBE cells by gob-5-specific antibody. Lane 1, western blot showing detection of gob-5 in granule membranes; lanes 2–4, western blots showing MARCKS-specific staining of isolated mucin granule membranes (2, control cells; 3, granule membranes from cells exposed to 100 μM RNS; 4, greatly diminished staining of granule membranes from cells exposed to 100 μM MANS).

Sections were embedded in 3–4% agar, then dehydrated and embedded in LR White resin (Electron Microscopy Sciences). Ultrathin (80–90 nm) sections were blocked with 10% FBS for 15 min, then incubated overnight at 4 °C with an affinity-purified rabbit antibody to mouse MARCKS protein (Abcam; 3 μg/ml), in PBS containing 0.5% BSA. After washing, grids were incubated with 12-nm gold–labeled secondary antibody (donkey anti-rabbit; Jackson ImmunoResearch Laboratories) in PBS containing 0.5% BSA for 2 h, then poststained with uranyl acetate.

Isolation of mucin granules from normal human bronchial epithelial (NHBE) cells. Intact mucin granules were isolated by a modification of the Wu and Castle method[17]. NHBE cells in primary air-liquid interface culture were collected 14 d after reaching confluence, lysed via sonication and centrifuged at 600*g* for 10 min. Postnuclear supernatants were diluted with 1.9 volumes of 86% Percoll, 0.3 M sucrose, 5 mM MOPS, 1 mM EDTA and 0.2 μg/ml of DPPD (pH 6.8) before centrifugation at 17,000*g* for 30 min. Crude mucin granules collected from the bottom 0.4 ml of the gradient were diluted and pelleted at 2,000*g* for 15 min, before overnight incubation at 4 °C in PBS containing 0.3 M sucrose with Dynal beads coated with a monoclonal antibody to mouse $CLCA_3$ (gob-5; an integral component of mucin granule membranes[18]). After incubation, the antibody-granule complex was eluted, boiled with 2× Laemmli sample buffer and applied to SDS-PAGE before immunoblotting using MARCKS- and $CLCA_3$-specific antibodies.

For immunoblotting, proteins were transferred to a polyvinylidene fluoride membrane, rinsed, blocked in 5% nonfat dry milk for 1 h, and incubated with primary antibody and 1% BSA overnight at 4 °C. After washing, the membrane was incubated with secondary antibody for 1 h. Biotinylated secondary antibody (Jackson ImmunoResearch Laboratories) and VECTASTAIN ABC (Vector Laboratories) were used to detect MARCKS. Proteins were detected with an Amersham ECL kit, followed by exposure to Hyperfilm ECL (Amersham Biosciences).

Statistical analysis. Results are presented as mean ± s.d. (n = 5–12 for each point, for ELISAs). Significance levels were calculated using one-way ANOVA, followed by the Scheffe test, using SPSS 6.1 software. $P < 0.05$ was considered significant.

ACKNOWLEDGMENTS

This study was supported by grant RO1-HL36982 from the National Institutes of Health (to K.B.A.). The authors thank P. Blackshear (National Institute of Environmental Health Sciences) for providing invaluable assistance and reagents, and S. Ho (University of Minnesota) for generously providing antibodies.

COMPETING INTERESTS STATEMENT

The authors declare competing financial interests (see the *Nature Medicine* website for details).

Received 3 October; accepted 16 December 2003
Published online at http://www.nature.com/naturemedicine/

1. Li, Y., Martin, L.D., Spizz, G. & Adler, K.B. MARCKS protein is a key molecule regulating mucin secretion by human airway epithelial cells *in vitro*. *J. Biol. Chem.* **276**, 40982–40990 (2001).
2. Eum, S.Y., Haile, S., Huerre, M. & Vargaftig, B.B. Eosinophil recruitment into the respiratory epithelium following antigenic challenge in hyper-IgE mice is accompanied by interleukin-5-dependent bronchial hyperresponsiveness. *Proc. Natl. Acad. Sci. USA* **92**, 12290–12294 (1995).
3. Haile, S. *et al.* Mucous cell metaplasia and inflammatory cell recruitment are dissociated in allergic mice following antibody- and drug-dependent cell depletion in a murine model of asthma. *Am. J. Respir. Cell Mol. Biol.* **20**, 891–902 (1999).
4. Singer, M., Lefort, J. & Vargaftig, B.B. Granulocyte depletion and dexamethasone differentially modulate airway hyperreactivity, inflammation, mucus accumulation, and secretion induced by rmIL-13 or antigen. *Am. J. Respir. Cell Mol. Biol.* **26**, 74–84 (2002).
5. Vargaftig, B.B. & Singer, M. Leukotrienes, IL-13, and chemokines cooperate to induce BHR and mucus in allergic mouse lungs. *Am. J. Physiol. Lung Cell. Mol. Physiol.* **284**, L260–L269 (2003).
6. Stumpo, D.J., Bock, C.B., Tuttle, J.S. & Blackshear, P.J. MARCKS deficiency in mice leads to abnormal brain development and perinatal death. *Proc. Nat. Acad. Sci. USA* **92**, 944–948 (1995).
7. Rose, S.D., Lejen, T., Zhang, L. & Trifaro, J.M. Chromaffin cell F-actin disassembly and potentiation of catecholamine release in response to protein kinase C activation by phorbol esters is mediated through myristoylated alanine-rich C kinase substrate phosphorylation. *J. Biol. Chem.* **276**, 36757–36763 (2001).
8. Elzagallaai, A., Rose, S.D. & Trifaro, J.M. Platelet secretion induced by phorbol ester stimulation is mediated through phosphorylation of MARCKS: a MARCKS-derived peptide blocks MARCKS phosphorylation and serotonin release without affecting pleckstrin phosphorylation. *Blood* **95**, 894–902 (2000).
9. Vaughan, P.F., Walker, J.H. & Peers, C. The regulation of neurotransmitter secretion by protein kinase C. *J. Mol. Neurobiol.* **18**, 125–155 (1998).
10. Trifaro, J., Rose, S.D., Lejen, T. & Elzagallaai, A. Two pathways control chromaffin cell cortical F-actin dynamics during exocytosis. *Biochimie* **82**, 339–352 (2000).
11. Salli, U., Supanic, S. & Stormshak, F. Phosphorylation of myristoylated alanine rich C kinase substrate (MARCKS) protein is associated with bovine luteal oxytocin exocytosis. *Biol. Reprod.* **63**, 12–20 (2000).
12. Hartness, M.E., Wade, J.A., Walker, J.H. & Vaughan, P.F. Overexpression of the myristoylated alanine-rich C kinase substrate decreases uptake and K^+-evoked release of noradrenaline in the human neuroblastoma SH-SY5Y. *Eur. J. Neurosci.* **13**, 925–934 (2001).
13. Seykora, J.T., Myatt, M.M., Allen, L.A.H., Ravetch, J.V. & Aderem, A. Molecular determinants of the myristoyl-electrostatic switch of MARCKS. *J. Biol. Chem.* **271**, 18797–18802 (1996).
14. Spizz, G. & Blackshear, P.J. Identification and characterization of cathepsin B as the cellular MARCKS cleaving enzyme. *J. Biol. Chem.* **272**, 23833–23842 (1997).
15. Shekels, L.L. *et al.* Mouse gastric mucin: cloning and chromosomal localization. *Biochem. J.* **311**, 775–785 (1995).
16. Vargaftig, B.B. & Singer, M. Leukotrienes mediate part of Ova-induced lung effects in mice via EGFR. *Am. J. Physiol. Lung Cell Mol. Physiol.* **285**, L808–L818 (2003).
17. Wu, T.T. & Castle, J.D. Evidence for colocalization and interaction between 37 and 39 kDa isoforms of secretory carrier membrane proteins (SCAMPs). *J. Cell Sci.* **110**, 1533–1541 (1997).
18. Leverkoehne, I. & Gruber, A.D. The murine mCLCA3(alias gob-5) protein is located in the mucin granule membranes of intestinal, respiratory and uterine goblet cells. *J. Histochem. Cytochem.* **50**, 829–838 (2002).

Attorney Docket No. 5051-451IP PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re: Li et al.
Serial No.: 09/914,020
Filed: December 31, 2001
For: *Methods and Compositions for Altering Mucus Secretion*

Confirmation No.: 8515
Art Unit: 1633
Examiner: J. Epps-Ford

Date: June 7, 2006

Mailstop Amendment
Commissioner for Patents
PO Box 1450
Alexandria, VA 22313-1450

ATTACHMENT C

Exhibit 8

Rogers et al. (2006) "Treatment of airway mucus hypersecretion," Annals of Medicine, 38:116-125.

Annals of Medicine. 2006; 38: 116–125

Taylor & Francis
Taylor & Francis Group

TRENDS IN CLINICAL PRACTICE

Treatment of airway mucus hypersecretion

DUNCAN F. ROGERS & PETER J. BARNES

Section of Airway Disease, National Heart & Lung Institute, Imperial College London, UK

Abstract

Airway mucus hypersecretion is now recognized as a key pathophysiological feature in many patients with asthma, chronic obstructive pulmonary disease (COPD) and cystic fibrosis. Consequently, it is important to develop drugs that inhibit mucus hypersecretion in these susceptible patients. Conventional therapies, including anticholinergics, β_2-adrenoceptor agonists, corticosteroids, mucolytics and macrolide antibiotics, have variable efficacy in inhibiting airway mucus hypersecretion, and are less effective in COPD than in asthma. Novel pharmacotherapeutic targets are being investigated, including inhibitors of nerve activity (e.g. large conductance calcium-activated potassium, BK_{Ca}, channel activators), tachykinin receptor antagonists, epoxygenase inducers (e.g. benzafibrate), inhibitors of mucin exocytosis (e.g. anti-myristoylated alanine-rich C kinase substrate (MARCKS), peptide and Munc-18B blockers), inhibitors of mucin synthesis and goblet cell hyperplasia (e.g. epidermal growth factor (EGF), receptor tyrosine kinase inhibitors, p38 mitogen-activated protein (MAP), kinase inhibitors, MAP kinase kinase/extracellular signal-regulated kinase (MEK/ERK), inhibitors, human calcium-activated chloride (hCACL2), channel blockers and retinoic acid receptor-α antagonists), inducers of goblet cell apoptosis (e.g. Bax inducers or Bcl-2 inhibitors), and purinoceptor P_{2Y2} antagonists to inhibit mucin secretion or P_{2Y2} agonists to hydrate secretions. However, real and theoretical differences delineate the mucus hypersecretory phenotype in asthma from that in COPD. More information is required on these differences to identify specific therapeutic targets which, in turn, should lead to rational design of anti-hypersecretory drugs for treatment of airway mucus hypersecretion in asthma and COPD.

Key words: *Asthma, chronic bronchitis, chronic obstructive pulmonary disease, COPD, epidermal growth factor, MUC gene, mucin, protease, protease inhibitor*

Introduction

Mucus secretion in the airways normally represents first-line defence of the respiratory tract and is an important part of innate immunity. However, if secretion becomes abnormal, mucus accumulates and may obstruct the airway lumen (Figure 1). Long-term and excessive mucus production, termed chronic mucus hypersecretion, can lead to significant airflow limitation in a number of severe respiratory conditions including asthma (1), chronic obstructive pulmonary disease (COPD) (2,3) and cystic fibrosis (CF) (4). It should be noted, however, that the impact of airway mucus hypersecretion on morbidity and mortality is not unequivocal or pertinent to all patients. Nevertheless, there are certain groups of patients, for example those with COPD who have mucus hypersecretion and are prone to chest infections (5), where a causal association has been demonstrated. Consequently, there is the perception that inhibiting mucus hypersecretion should have clinical benefit in hypersecretory conditions of the airways. Numerous pharmaceutical and other treatments are available or in development that are aimed, either directly or indirectly, at inhibiting mucus hypersecretion. However, although asthma and COPD share mucus obstruction as a clinical feature, the pathophysiological mechanisms underlying the impairment in mucus clearance may be different, to a greater or lesser extent, for each condition. For example, there are real and theoretical differences in the pathophysiology of airway mucus hypersecretion between asthma and COPD (Figure 2). In addition, the features of airway inflammation and remodelling that uniquely characterize different respiratory diseases

Correspondence: Dr D. F. Rogers, Thoracic Medicine, National Heart & Lung Institute, Imperial College London, Dovehouse Street, London SW3 6LY, UK. Fax: +44 20 7351 8126. E-mail: duncan.rogers@imperial.ac.uk

ISSN 0785-3890 print/ISSN 1365-2060 online
DOI: 10.1080/07853890600585795

are also factors that influence mucus hypersecretion. Consequently, no single therapy is likely to be effective across the spectrum of mucus obstructive conditions of the respiratory tract. It is possible that disease specific therapy will be more successful.

The present review discusses airway mucus hypersecretion, using asthma and COPD as examples of respiratory conditions that share excess airway mucus as a clinical feature but with specific differences in mucus pathophysiology. Cystic fibrosis will be briefly mentioned where appropriate for comparative purposes as a third respiratory condition with its own pathophysiology and mucus problems. However, the mucus problems in CF pertain predominantly to the genetic defect in the cystic fibrosis transmembrane conductance regulator (CFTR) and the associated abnormality in sodium channels, and are beyond the scope of the present article. The interested reader is referred to two recent reviews (4,6). Factors involved in the rational design of pharmacotherapeutic compounds to inhibit airway mucus hypersecretion in asthma and COPD will be considered. A brief description of airway mucus is given first.

Key messages

- Airway mucus hypersecretion is now recognized as an important contributor to morbidity and mortality in many patients with severe chronic lung diseases.
- The airway mucus hypersecretory phenotypes of asthma, COPD and cystic fibrosis have many important differences.
- Effective novel pharmacotherapy of airway mucus hypersecretion may have to be disease specific.

Airway mucus, mucins and MUC genes

Airway luminal mucus is a complex dilute aqueous solution of lipids, glycoconjugates and proteins. It comprises salts, enzymes and anti-enzymes, oxidants and antioxidants, exogenous bacterial products, endogenous antibacterial agents, cell-derived mediators and proteins, plasma-derived mediators and proteins, and cell debris such as DNA. Airway mucus is considered to form a liquid bi-layer whereby an upper gel layer floats above a lower, more watery sol, or periciliary liquid, layer (6). The gel layer traps particles and is moved on the tips of the cilia. Respiratory tract mucus requires the correct combination of viscosity and elasticity for optimal efficiency of ciliary interaction. Viscoelasticity is conferred on the mucus primarily by high molecular weight mucous glycoproteins, termed mucins, which comprise up to 2% by weight of the mucus (7). In the airways, mucins are produced by goblet cells in the epithelium (8) and sero-mucous glands in the submucosa (9). Mucins are thread-like molecules comprising a linear peptide sequence (termed apomucin), often with tandemly repeated regions, that is highly glycosylated, predominantly via *O*-linkages (Figure 3). Apomucins are encoded by specific mucin (MUC) genes, with 19 human MUC genes currently recognized, namely MUC1, 2, 3A, 3B, 4, 5AC, 5B, 6–9, 11–13 and 16–20 (10–15). Of these, however, it is only the MUC5AC and MUC5B gene products that comprise the major gel-forming mucins in respiratory

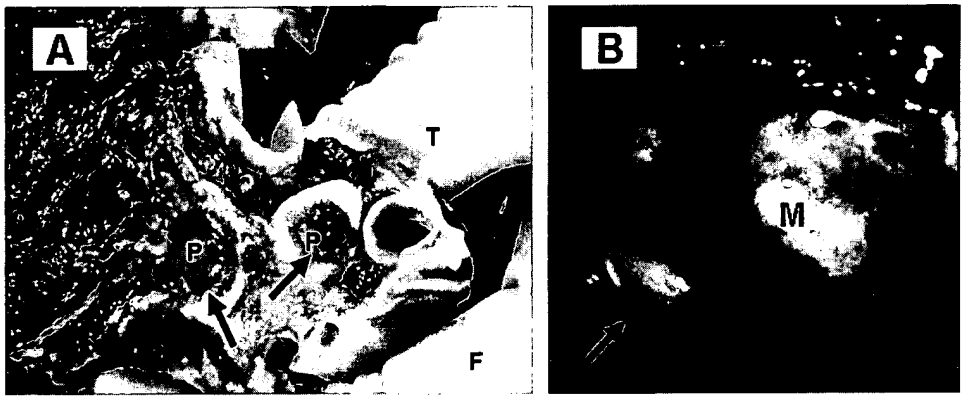


Figure 1. Mucus obstruction of the airways in asthma and COPD: gross pathology. A: Lung from an asthmatic patient cut through to show gelatinous plugs (P) in the large airways (arrows). Courtesy Dr Catherine Corbishley (thumb, T, and finger, F, holding specimen). B: Luminal mucus (M) partially blocking an extrapulmonary bronchus (arrow) in a long-term elderly male cigarette smoker with chronic sputum production.

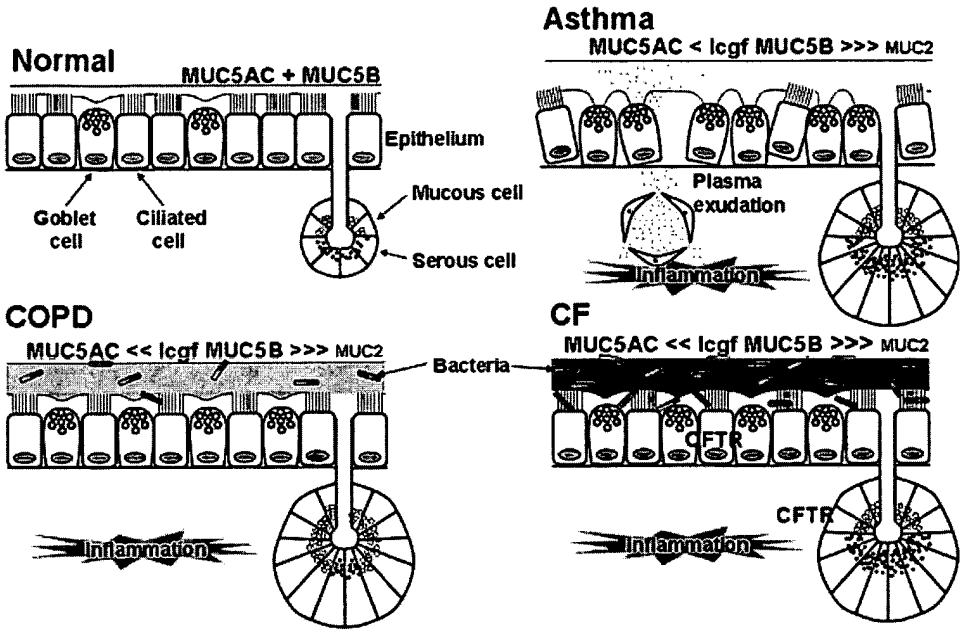


Figure 2. Putative differences in pathophysiology of the airway mucus hypersecretory phenotype in asthma, chronic obstructive pulmonary disease (COPD) and cystic fibrosis (CF). Compared with normal, in asthma there is airway inflammation, increased luminal mucus, with an increased ratio of MUC5B (low charge glycoform (lcgf)) to MUC5AC, possibly small amounts of MUC2 present in the mucus, epithelial 'fragility' with loss of ciliated cells, marked goblet cell hyperplasia, submucosal gland hypertrophy (although without a marked increase in mucous to serous ratio), 'tethering' of mucus to goblet cells, and plasma exudation. In COPD, there is airway inflammation, increased luminal mucus, goblet cell hyperplasia, submucosal gland hypertrophy (with an increased proportion of mucous to serous acini), an increased ratio of lgcf MUC5B to MUC5AC above that in asthma, possibly small amounts of MUC2 in the mucus, and a susceptibility to infection. In CF, there is airway inflammation, increased luminal mucus, goblet cell hyperplasia, submucosal gland hypertrophy, an increased ratio of lgcf MUC5B to MUC5AC, small amounts of MUC2 in the mucus, and a marked susceptibility to infection. Many of these differences require confirmation (or otherwise) by data from greater numbers of subjects.

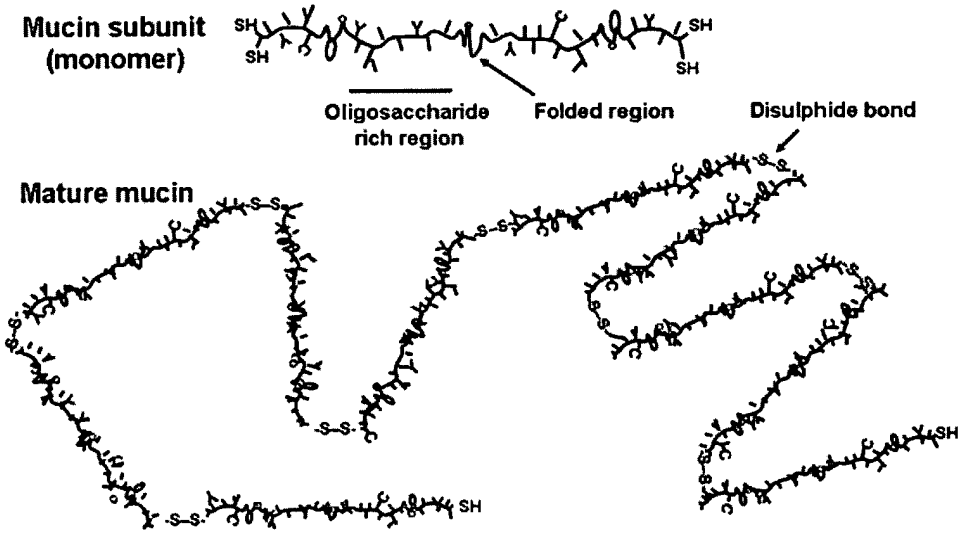


Figure 3. Schematic representation of a gel-forming mucin molecule. The mucin subunit (~500 nm in length) comprises an amino acid backbone with highly glycosylated areas and folded regions, stabilized via disulphide bonds, with little or no glycosylation. Glycosylation is via *O*-linkages and is highly diverse. In secretions, the subunits are joined end-to-end by disulphide bonds (S-S) into long thread-like mature mucin molecules.

secretions from normal subjects. The mucin content of secretions from patients with hypersecretory respiratory diseases may differ from normal (see below).

Mucus obstruction of the airways in asthma and COPD

Asthma and COPD are chronic, severe inflammatory conditions of the airways in which mucus hypersecretion is a pathophysiological feature. The increase in airway luminal mucus is associated with increases in amount of mucus-secreting tissue.

Asthma

Asthma is a chronic inflammatory condition of the airways that has specific clinical and pathophysiological features (16), including mucus obstruction of the airways (17) (Figure 2). The latter is particularly evident in a proportion of patients who die in *status asthmaticus*, where many airways are occluded by tenacious mucus plugs (18–20). There is also more mucus in the central and peripheral airways of both chronic and severe asthmatics compared with control subjects (21). The increased amount of luminal mucus reflects an increase in amount of airway secretory tissue, due to both goblet cell hyperplasia (21,22), and submucosal gland hypertrophy (19), although the latter is not characteristic of all patients with asthma (21).

COPD

COPD comprises three overlapping conditions, namely chronic obstructive bronchitis (commonly associated with increased airway mucus hypersecretion), chronic bronchiolitis (small airways disease) and emphysema (airspace enlargement due to alveolar destruction) (23). The following discussion considers the 'bronchitic' component of COPD. The airways of patients with COPD contain excessive amounts of mucus (24), which is markedly increased above that in control subjects (25,26) (Figure 2). The increased mucus is linked specifically with an increase in MUC5B in the bronchiolar lumen (27). The excessive luminal mucus is associated with increased amounts of mucus-secreting tissue, namely goblet cell hyperplasia (24,28,29) and submucosal gland hypertrophy (24,25,30,31). The amount of gland correlates with the amount of luminal mucus (25).

Differences in pathophysiology relating to mucus obstruction in asthma, COPD and CF

In order to develop appropriate models of airway mucus hypersecretion and develop drugs to inhibit hypersecretion, it is necessary to understand the similarities and differences in the features of mucus obstruction for different hypersecretory conditions. There are a number of differences in the pathophysiology of mucus hypersecretion between asthma, COPD and CF (Figure 2). Firstly, although the underlying pulmonary inflammation of asthma and COPD share many common features, there are specific characteristics unique to each condition (32–35). Asthma is invariably an allergic disease that affects the airways, rather than the lung parenchyma, and is characterized by Th2-lymphocytic orchestration of pulmonary eosinophilia. In contrast, COPD is currently perceived as predominantly a neutrophilic disorder governed largely by macrophages and epithelial cells. It is associated primarily with cigarette smoking. The differences between asthma and COPD in their profile of airway inflammation and remodelling may in turn exert different influences on the development of airway mucus obstruction in the two conditions.

Airway mucus in asthma is more viscous than in COPD or CF, with the airways of asthmatic patients tending to develop, and subsequently become blocked by, gelatinous 'mucus' plugs (17). Whether or not mucus in asthma has an intrinsic biochemical abnormality is unclear. In general terms, sputum from patients with asthma is more viscous than that from patients with chronic bronchitis or bronchiectasis (36–38). Mucus plugs in asthma differ from airway mucus gels in chronic bronchitis and CF in that they are stabilized by non-covalent interactions between extremely large mucins assembled from conventionally sized subunits (39). This suggests an intrinsic defect in assembly of the mucin molecules, and could account for the increased viscosity of the mucus plugs in asthma. Plug formation may also be due, at least in part, to increased airway plasma exudation in asthma compared with COPD (40). In addition, and in direct contrast to COPD, exocytosed mucins in asthma are not released fully from the goblet cells, leading to 'tethering' of luminal mucins to the airway epithelium (41). This tethering may also contribute to plug formation. One explanation of mucus tethering is that, in COPD, neutrophil proteases cleave goblet cell-attached mucins, whereas in asthma the inflammatory cell profile, predominantly an eosinophilia, does not generate the appropriate proteases to facilitate mucin release.

Different MUC gene products may be present in respiratory secretions in asthma and COPD. MUC5AC and a low-charge glycoform of MUC5B are the major mucin species in airway secretions from patients with asthma, COPD and CF (42–46). There is significantly more of the low-charge glycoform of MUC5B in the respiratory diseases than in normal control secretions (46). Interestingly, there is a proportional increase in the MUC5B mucin over the MUC5AC mucin in airway secretions from patients with COPD or CF, compared with secretions from patients with asthma (42). The significance of the change in MUC5B glycoforms to bacterial colonization between the different diseases is unclear. However, it is interesting that it is observed in COPD and CF, where patients are prone to bacterial chest infections (4,23), rather than in asthma where patients are not notably prone to infection.

In contrast to normal airways, there is upregulation of MUC5AC in the airway epithelium of patients with COPD compared with smoking (and non-smoking) controls (27). Goblet cells in the airways from patients with COPD contain not only MUC5AC but also MUC5B (44,47) and MUC2 (7,48). The latter distribution is different to that in the airways of patients with asthma or CF, where MUC5AC and MUC5B show a similar localization to that in normal subjects (49,50). It is noteworthy that although MUC2 is located in goblet cells in irritated airways, and MUC2 mRNA is found in the airways of smokers (26), MUC2 mucin is either not found in airway secretions from normal subjects or patients with chronic bronchitis (43), or is found only in very small amounts in asthma, COPD and CF (46,51). The significance of the above combined observations is unclear, but suggests that there are differences in goblet cell phenotype between asthma and COPD.

Another notable difference between asthma and COPD is in the bronchial submucosal glands (52). In asthma, although hypertrophied, the glands are morphologically normal with an even distribution of mucous and serous cells. In contrast, in chronic bronchitis, gland hypertrophy is characterized by an increased number of mucous cells relative to serous cells, particularly in severe bronchitis (52).

From the above, it may be seen that there are theoretical and actual differences in the nature of airway mucus between asthma and COPD. How these differences relate to pathophysiology and clinical symptoms in the two conditions is, for the most part, unclear. However, these dissimilarities suggest that different treatments may be required for effective treatment of airway mucus obstruction in asthma and COPD.

Pharmacotherapy of airway mucus hypersecretion

The prevalence of patient presentation with cough and expectoration of sputum and the perceived importance of mucus in the pathophysiology of severe lung conditions, such as asthma and COPD, have led to development of drugs intended to treat airway mucus hypersecretion (53). There are two objectives for treatment of airway mucus obstruction: short-term relief of symptoms and long-term benefit (Table I). Pharmacotherapy of airway mucus hypersecretion in asthma and COPD has been discussed in detail recently (17,54) and is summarized below and in Table II. Essentially, pharmacotherapy divides into two sections. The first is anti-inflammatory treatment of airway inflammation, probably the most beneficial therapy overall. The second is therapies directed specifically at different aspects of the pathophysiology of mucus

Table I. Objectives for effective pharmacotherapy of mucus pathophysiology in chronic airway hypersecretory diseases.

Overall objective	Component objective
Facilitate mucus clearance (short-term relief of symptoms)	Reduce viscosity (? increase elasticity)
	Increase ciliary function
	Induce cough
	Facilitate release of 'tethered' goblet cell mucin (asthma)
	Treat pulmonary infection (COPD)
Reverse hypersecretory phenotype (long-term benefit)	Treat airway/pulmonary inflammation
	Reduce goblet cell number
	Reduce submucosal gland size
	Correct increased gland mucous:serous cell ratio (COPD)
	Reverse increased MUC5B:MUC5AC ratio (especially in COPD)
	Inhibit plasma exudation (asthma)
	Inhibit production of MUC2
	Inhibit development of epithelial 'fragility' (asthma)

Table II. Potential therapeutic targets and inhibitors of airway mucus hypersecretion.

Target	Inhibitor(s)
Airway inflammation	Glucocorticosteroids, PDE4 inhibitors, suplatast tosilate, cytokine/chemokine blockers/receptor antagonists, ?macrolide antibiotics
Mucus properties:	
Thickened mucus	Mucolytic drugs (e.g. *N*-acetylcysteine)
$P2Y_2$ receptors	Selective agonists for mucus hydration (e.g. INS37217)
Goblet cell hyperplasia:	
Bcl-2	Antisense oligonucleotides (e.g. G3139, aka Genasense, oblimersen), Bax mimetics
hCLCA1	Talniflumate
EGFR tyrosine kinase	AG1478, BIBX1522, ZD1839 (Iressa)
ERK	MEK inhibitors (e.g. PD98059, U0126)
MUC gene expression	18-mer MUC antisense oligonucleotide
NKCC1	Bumetanide
p38 MAPK	p38 MAPK inhibitors (e.g. SB 203580)
PI-3K	PI-3K inhibitors (e.g. LY-294002)
RAR-α	RAR-α antagonists (e.g. RO-41-5253)
Inflammatory mediators:	
Bradykinin (B_2 receptors)	Icatibant
Cysteinyl leukotrienes (Cys-LT_1 receptors)	Montelukast, zafirlukast
Endothelin-1 (ET_A receptors)	Bosentan
Mast cell tryptase	APC-366, BABIM
Neutrophil elastase	Elastase inhibitors (e.g. batimastat, suramin and macrolide antibiotics such as erythromycin and flurythromycin)
PAF	Apafant, modipafant
Purine nucleotides (e.g. ATP, UTP)	$P2Y_2$ antagonists (none yet available)
Mucin exocytosis:	
MARCKS	MARCKS inhibitors (e.g. MANS peptide)
Munc-18B	Munc-18B inhibitors (antisense oligomer)
SNARE proteins	Re-targeted clostridial endopeptidase fusion proteins (e.g. EGF-LH_N-C)
Neural pathways:	
Muscarinic (M_3) receptors	Anticholinergics (e.g. ipratropium bromide, tiotropium bromide)
Nerve activation	VR-1 receptor antagonists (e.g. anandamide, capsazepine)
Neurotransmitter release	BK_{Ca} channel activators (e.g. NS 1619), CB_2 receptor agonists (e.g. AM1241, SR144528)
Tachykinin NK_1 receptors	Tachykinin NK_1 receptor antagonists (e.g. CP99,994, RP67580, nolpitantium; dual NK_1/NK_2 and triple $NK_1/NK_2/NK_3$ antagonists)

Abbreviations: aka=also known as; BABIM=bis(5-amidino-2-benzimidazo-lyl)methane; Bax=endogenous pro-apoptotic factor; Bcl-2=endogenous antiapoptotic factor; CB=cannabinoid (receptor); EGFR=epidermal growth factor receptor; ERK=extracellular signal-regulated kinase; MAPK=mitogen activated protein kinase; MARCKS=myristoylated alanine-rich C kinase substrate; MEK=MAPK kinase ('upstream' of ERK); MUC=mucin; NK=neurokinin; NKCC=Na^+-K^+-Cl^- cotransporter; PAF=platelet activating factor; PI-3K=phosphatidylinositol 3-kinase; PDE=phosphodiesterase; RAR=retinoic acid receptor; SNARE=soluble N-ethyl-maleimide-sensitive factor attachment protein receptor; VR=vanilloid receptor.

hypersecretion, from inhibition of the secretagogue effects of specific endogenous inflammatory secretagogues to aiding expectoration of sputum by use of mucolytics.

Conventional pharmacotherapy

The medications used currently in clinical management of COPD and asthma, namely bronchodilators (anticholinergics, β_2-adrenoceptor agonists and methylxanthines) and anti-inflammatories (primarily glucocorticosteroids), are not administered necessarily to target airway hypersecretion, but may nevertheless exert some of their beneficial effects via actions on mucus (55).

Decreasing the viscosity of airway mucus with mucolytic drugs should improve mucus clearance, both by mucociliary transport and by cough. However, although numerous mucolytic drugs are available worldwide, their effectiveness in treatment of asthma and COPD has not been clearly established (56). Similarly, although antibiotics are recommended in clinical management of exacerbations of COPD, there is no evidence for prophylactic antibiotic treatment of stable COPD (23,57).

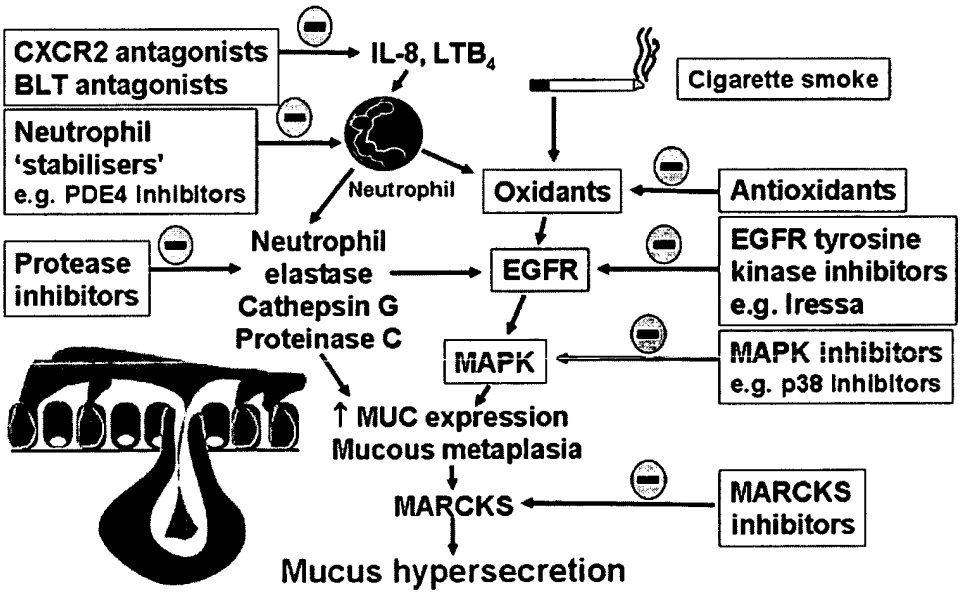


Figure 4. Possibilities for novel inhibition of airway mucus hypersecretion in chronic obstructive pulmonary disease (COPD). Theoretically, recruitment and activation of neutrophils and inhalation of cigarette smoke increases the lung burden of proteases and oxidants, which in turn activate the epidermal growth factor (EGF) intracellular signalling pathway leading to increased mucin (MUC) gene expression and mucin synthesis, mucous metaplasia (goblet cell hyperplasia and submucosal gland hypertrophy) and increased mucin secretion (via myristoylated alanine-rich C kinase substrate (MARCKS)). The neutrophil chemotactic activity of interleukin (IL)-8 and leukotriene (LT) B_4 can be inhibited by selective antagonists at the receptors for CXC chemokines (CXCR2 antagonists) and for LTB_4 (BLT antagonists), respectively, and neutrophil activity can be inhibited by inhibitors of phosphodiesterase (PDE) 4. The activity of proteases and oxidants can be inhibited by protease inhibitors and antioxidants. The EGF receptor and mitogen activated protein kinase cascade, as well as MARCKS activity, can be inhibited by appropriate inhibitors.

Novel pharmacotherapy

There is a range of novel pharmacotherapeutics in development for inhibition of airway mucus hypersecretion, including inhibitors of mucin exocytosis and inhibitors of goblet cell hyperplasia (Figure 4).

Exocytosis inhibitors

Inhibition of mucin exocytosis is a therapeutic option for mucus hypersecretion in asthma and COPD. Myristoylated alanine-rich C kinase substrate (MARCKS) protein is a key intracellular molecule involved in intracellular movement and exocytosis of mucin granules (58). Blockade of MARCKS by a synthetic peptide to its N-terminal region (MANS peptide) inhibited mucin secretion by normal human bronchial epithelial cells *in vitro* (58) and by mouse airway epithelium *in vivo* (59). The Sec1/Munc18 family are critical to exocytosis in airway goblet cells, and experimental induction of Munc18B (mammalian homologue of the nematode *unc-18* gene product, also known as syntaxin-binding protein 1) induces a marked airway hypersecretory phenotype (60). *Clostridium botulinum* neurotoxins (BoNT) inhibit neurotransmitter (e.g. acetylcholine) release by cleaving soluble N-ethyl-maleimide-sensitive factor attachment protein receptor (SNARE) proteins in the nerve terminal (61). Conversely, BoNTs could be retargeted to airway secretory cells via a fusion ligand to selectively inhibit mucin exocytosis, and thereby reduce mucus output. For example, the LH_N fragment (representing the light chain and the N-terminal domain of the heavy chain coupled via a single disulphide bond) of BoNT-C fused to EGF (termed EGF-LH_N-C) as targeting ligand for the EGF-R on airway epithelial cells markedly inhibits stimulated mucin output from a human respiratory epithelial cell line (62).

Inhibition of mucin synthesis and goblet cell hyperplasia

Increased MUC gene expression, mucin synthesis and goblet cell hyperplasia appear to be linked processes that are regulated by a number of inflammatory mechanisms. For example, airway epidermal growth factor receptor (EGF-R) upregulation and signalling via EGF-R tyrosine kinase is a key signalling event for induction of mucin synthesis and goblet cell hyperplasia (63,64). Inhibitors of EGF-R tyrosine kinase block these responses. One of these, gefitinib (Iressa), is in clinical trial for cancer, but not yet for asthma or COPD.

The p38 mitogen-activated protein (MAP) kinase pathway, the MEK/ERK pathway, and the phosphatidylinositol 3-kinase pathway are all involved, to a greater or lesser extent, in intracellular events leading to mucin synthesis and goblet cell hyperplasia (65–68). Inhibitors of these pathways inhibit mucus hypersecretory endpoints in experimental systems.

Calcium-activated chloride (CLCA) channels appear to be critically involved in development of an airway hypersecretory phenotype (69). In mice, suppression of mCLCA3 inhibits goblet cell hyperplasia, whilst overexpression increases goblet cell number (70). Talniflumate is a small molecule putative inhibitor of hCLCA1 which is currently being developed as a mucoregulatory treatment for asthma and COPD (71).

Retinoic acid (vitamin A) acting on the RAR-α receptor appears to be involved in mucin expression (72–74) and in the development and maintenance of a hypersecretory phenotype (74). RAR-α antagonists, such as RO-41-5253, inhibit a number of these activities (75).

Antisense technology is also being explored as a new approach to inhibition of goblet cell hyperplasia. For example, an 18-mer MUC antisense oligomer suppressed mucin gene expression and wood smoke-induced epithelial metaplasia in rabbit airways (76).

Hyperplastic airway goblet cells in COPD models express the antiapoptotic factor Bcl-2 (77). Reduction of Bcl-2 expression by antisense oligonucleotides induces a dose-dependent resolution of hyperplasia.

P_{2Y2} agonists and antagonists

Adenosine 5'-triphosphate (ATP) and uridine triphosphate (UTP) increase airway mucin and water secretion via interaction with P_{2Y2} purinoceptors (78,79). Consequently, P_{2Y2} antagonists might inhibit airway hypersecretion (80). However, mucus hydration is associated with improved mucociliary clearance, and stimulation of water secretion may have greater therapeutic potential than inhibition of P_{2Y2}-mediated mucin secretion (6). Consequently, there is considerable interest in development of P_{2Y2} agonists (78).

Conclusions

Mucus secretion in the airways is a vital homeostatic mechanism that protects the respiratory tract from a barrage of inhaled insult. However, abnormal production of mucus can contribute to respiratory disease. Airway obstruction by mucus is a common feature of a number of severe respiratory conditions, including asthma, COPD and CF. These diseases share pulmonary inflammation and remodelling as a pathophysiological characteristic. They also each have a number of unique features that characterize their airway mucus obstruction. For example, mucus plug formation and mucus tethering are features of asthma, whereas submucosal gland hypertrophy with a disproportionate increase in the ratio of mucous to serous cells is a significant feature in COPD. Understanding of the relative importance of the differences and similarities in the pathophysiology of mucus obstruction between different respiratory diseases should lead to rational development of therapeutic interventions.

References

1. Del Donno M, Bittesnich D, Chetta A, Olivieri D, Lopez-Vidriero MT. The effect of inflammation on mucociliary clearance in asthma: an overview. Chest. 2000;118:1142–9.
2. Houtmeyers E, Gosselink R, Gayan-Ramirez G, Decramer M. Regulation of mucociliary clearance in health and disease. Eur Respir J. 1999;13:1177–88.
3. Maestrelli P, Saetta M, Mapp CE, Fabbri LM. Remodeling in response to infection and injury. Airway inflammation and hypersecretion of mucus in smoking subjects with chronic obstructive pulmonary disease. Am J Respir Crit Care Med. 2001;164:S76–S80.
4. Davis PB. Cystic fibrosis. Pediatr Rev. 2001;22:257–64.
5. Prescott E, Lange P, Vestbo J. Chronic mucus hypersecretion in COPD and death from pulmonary infection. Eur Respir J. 1995;8:1333–8.
6. Knowles MR, Boucher RC. Mucus clearance as a primary innate defense mechanism for mammalian airways. J Clin Invest. 2002;109:571–7.
7. Davies JR, Herrmann A, Russell W, Svitacheva N, Wickström C, Carlstedt I. Respiratory tract mucins: structure and expression patterns. Novartis Foundation Symposium 248. Mucus Hypersecretion in Respiratory Disease. Chichester: John Wiley & Sons, 2002, pp. 76–93.
8. Rogers DF. The airway goblet cell. Int J Biochem Cell Biol. 2003;35:1–6.
9. Finkbeiner WE. Physiology and pathology of tracheobronchial glands. Respir Physiol. 1999;118:77–83.
10. Moniaux N, Escande F, Porchet N, Aubert JP, Batra SK. Structural organization and classification of the human mucin genes. Front Biosci. 2001;6:D1192–D1206.
11. Dekker J, Rossen JW, Buller HA, Einerhand AW. The MUC family: an obituary. Trends Biochem Sci. 2002;27:126–31.
12. Gum JR Jr, Crawley SC, Hicks JW, Szymkowski DE, Kim YS. MUC17, a novel membrane-tethered mucin. Biochem Biophys Res Commun. 2002;291:466–75.
13. Wu GJ, Wu MW, Wang SW, Liu Z, Qu P, Peng Q, et al. Isolation and characterization of the major form of human MUC18 cDNA gene and correlation of MUC18 overexpression in prostate cancer cell lines and tissues with malignant progression. Gene. 2001;279:17–31.
14. Chen Y, Zhao YH, Kalaslavadi TB, Hamati E, Nehrke K, Le AD, et al. Genome-wide search and identification of a novel gel-forming mucin MUC19/Muc19 in glandular tissues. Am J Respir Cell Mol Biol. 2004;30:155–65.

15. Higuchi T, Orita T, Nakanishi S, Katsuya K, Watanabe H, Yamasaki Y, et al. Molecular cloning, genomic structure, and expression analysis of MUC20, a novel mucin protein, up-regulated in injured kidney. J Biol Chem. 2004;279:1968–79.
16. Eapen SS, Busse WW. Asthma. Clin Allergy Immunol. 2002;16:325–53.
17. Rogers DF. Airway mucus hypersecretion in asthma: an undervalued pathology? Curr Opin Pharmacol. 2004; 4: 241–50.
18. Houston JC, De Navasquez S, Trounce JR. A clinical and pathological study of fatal cases of status asthmaticus. Thorax. 1953;8:207–13.
19. Dunnill MS. The pathology of asthma with special reference to changes in the bronchial mucosa. J Clin Pathol. 1960;13: 27–33.
20. Saetta M, Di Stefano A, Rosina C, Thiene G, Fabbri LM. Quantitative structural analysis of peripheral airways and arteries in sudden fatal asthma. Am Rev Respir Dis. 1991; 143:138–43.
21. Aikawa T, Shimura S, Sasaki H, Ebina M, Takishima T. Marked goblet cell hyperplasia with mucus accumulation in the airways of patients who died of severe acute asthma attack. Chest. 1992;101:916–21.
22. Ordonez CL, Khashayar R, Wong HH, Ferrando R, Wu R, Hyde DM, et al. Mild and moderate asthma is associated with airway goblet cell hyperplasia and abnormalities in mucin gene expression. Am J Respir Crit Care Med. 2001;163:517–23.
23. National Heart Lung and Blood Institute, World Health Organisation, Global initiative for chronic obstructive lung disease. Publication number 2701. Bethesda National Institutes of Health; 2001.
24. Reid L. Pathology of chronic bronchitis. Lancet. 1954;266: 274–8.
25. Aikawa T, Shimura S, Sasaki H, Takishima T, Yaegashi H, Takahashi T. Morphometric analysis of intraluminal mucus in airways in chronic obstructive pulmonary disease. Am Rev Respir Dis. 1989;140:477–82.
26. Steiger D, Fahy J, Boushey H, Finkbeiner WE, Basbaum C. Use of mucin antibodies and cDNA probes to quantify hypersecretion in vivo in human airways. Am J Respir Cell Mol Biol. 1994;10:538–45.
27. Caramori G, Di Gregorio C, Carlstedt I, Casolari P, Guzzinati I, Adcock IM, et al. Mucin expression in peripheral airways of patients with chronic obstructive pulmonary disease. Histopathology. 2004;45:477–84.
28. Saetta M, Turato G, Baraldo S, Zanin A, Braccioni F, Mapp CE, et al. Goblet cell hyperplasia and epithelial inflammation in peripheral airways of smokers with both symptoms of chronic bronchitis and chronic airflow limitation. Am J Respir Crit Care Med. 2000;161:1016–21.
29. Mullen JB, Wright JL, Wiggs BR, Pare PD, Hogg JC. Structure of central airways in current smokers and ex-smokers with and without mucus hypersecretion: relationship to lung function. Thorax. 1987;42:843–8.
30. Reid L. Measurement of the bronchial mucous gland layer: a diagnostic yardstick in chronic bronchitis. Thorax. 1960;15: 132–41.
31. Restrepo G, Heard BE. The size of the bronchial glands in chronic bronchitis. J Pathol Bacteriol. 1963;85:305–10.
32. Jeffery PK. Differences and similarities between chronic obstructive pulmonary disease and asthma. Clin Exp Allergy. 1999;29 Suppl 2:14–26.
33. Saetta M, Turato G, Maestrelli P, Mapp CE, Fabbri LM. Cellular and structural bases of chronic obstructive pulmonary disease. Am J Respir Crit Care Med. 2001;163: 1304–9.
34. Djukanovic R. Airway inflammation in asthma and its consequences: implications for treatment in children and adults. J Allergy Clin Immunol. 2002;109 Suppl 6: S539–S548.
35. Barnes PJ. Mediators of chronic obstructive pulmonary disease. Pharmacol Rev. 2004;56:515–48.
36. Lopez-Vidriero MT, Reid L. Chemical markers of mucous and serum glycoproteins and their relation to viscosity in mucoid and purulent sputum from various hypersecretory diseases. Am Rev Respir Dis. 1978;117:465–77.
37. Charman J, Reid L. Sputum viscosity in chronic bronchitis, bronchiectasis, asthma and cystic fibrosis. Biorheology. 1972; 9:185–99.
38. Shimura S, Sasaki T, Sasaki H, Takishima T, Umeya K. Viscoelastic properties of bronchorrhoea sputum in bronchial asthmatics. Biorheology. 1988;25:173–9.
39. Sheehan JK, Richardson PS, Fung DC, Howard M, Thornton DJ. Analysis of respiratory mucus glycoproteins in asthma: a detailed study from a patient who died in status asthmaticus. Am J Respir Cell Mol Biol. 1995;13:748–56.
40. Rogers DF, Evans TW. Plasma exudation and oedema in asthma. Br Med Bull. 1992;48:120–34.
41. Shimura S, Andoh Y, Haraguchi M, Shirato K. Continuity of airway goblet cells and intraluminal mucus in the airways of patients with bronchial asthma. Eur Respir J. 1996;9: 1395–401.
42. Thornton DJ, Carlstedt I, Howard M, Devine PL, Price MR, Sheehan JK. Respiratory mucins: identification of core proteins and glycoforms. Biochem J. 1996;316:967–75.
43. Hovenberg HW, Davies JR, Herrmann A, Linden CJ, Carlstedt I. MUC5AC, but not MUC2, is a prominent mucin in respiratory secretions. Glycoconj J. 1996;13: 839–47.
44. Wickstrom C, Davies JR, Eriksen GV, Veerman EC, Carlstedt I. MUC5B is a major gel-forming, oligomeric mucin from human salivary gland, respiratory tract and endocervix: identification of glycoforms and C-terminal cleavage. Biochem J. 1998;334:685–93.
45. Sheehan JK, Howard M, Richardson PS, Longwill T, Thornton DJ. Physical characterization of a low-charge glycoform of the MUC5B mucin comprising the gel-phase of an asthmatic respiratory mucous plug. Biochem J. 1999;338:507–13.
46. Kirkham S, Sheehan JK, Knight D, Richardson PS, Thornton DJ. Heterogeneity of airways mucus: variations in the amounts and glycoforms of the major oligomeric mucins MUC5AC and MUC5B. Biochem J. 2002;361:537–46.
47. Chen Y, Zhao YH, Di YP, Wu R. Characterization of human mucin 5B gene expression in airway epithelium and the genomic clone of the amino-terminal and 5′-flanking region. Am J Respir Cell Mol Biol. 2001;25:542–53.
48. Davies JR, Carlstedt I. Respiratory tract mucins. In: Salthe M, editor. Cilia and Mucus: from Development to Respiratory Defense. New York: Marcel Dekker, Inc., 2001. p. 167–78.
49. Groneberg DA, Eynott PR, Lim S, Oates T, Wu R, Carlstedt I, et al. Expression of respiratory mucins in fatal status asthmaticus and mild asthma. Histopathology. 2002; 40:367–73.
50. Groneberg DA, Eynott PR, Oates T, Lim S, Wu R, Carlstedt I, et al. Expression of MUC5AC and MUC5B mucins in normal and cystic fibrosis lung. Respir Med. 2002;96:81–6.
51. Davies JR, Svitacheva N, Lannefors L, Kornfalt R, Carlstedt I. Identification of MUC5B, MUC5AC and small amounts of MUC2 mucins in cystic fibrosis airway secretions. Biochem J. 1999;344:321–30.

52. Glynn AA, Michaels L. Bronchial biopsy in chronic bronchitis and asthma. Thorax. 1960;15:142–53.
53. Houtmeyers E, Gosselink R, Gayan-Ramirez G, Decramer M. Effects of drugs on mucus clearance. Eur Respir J. 1999;14: 452–67.
54. Rogers DF. The role of airway secretions in COPD: pathophysiology, epidemiology and pharmacotherapeutic options. COPD: J Chron Obstructive Pulm Dis. 2005;2: 341–53.
55. Rogers DF. Mucociliary dysfunction in COPD: effect of current pharmacotherapeutic options. Pulm Pharmacol Ther. 2005;18:1–8.
56. Rogers DF. Mucoactive drugs for asthma and COPD: any place in therapy? Expert Opin Investig Drugs. 2002;11: 15–35.
57. National Institute for Clinical Excellence (NICE), Chronic obstructive pulmonary disease: national clinical guideline on management of chronic obstructive pulmonary disease in adults in primary and secondary care. Thorax. 2004;59(Suppl 1): 1–232.
58. Li Y, Martin LD, Spizz G, Adler KB. MARCKS Protein Is a Key Molecule Regulating Mucin Secretion by Human Airway Epithelial Cells in Vitro. J Biol Chem. 2001;276:40982–90.
59. Singer M, Martin LD, Vargaftig BB, Park J, Gruber AD, Li Y, et al. A MARCKS-related peptide blocks mucus hypersecretion in a mouse model of asthma. Nat Med. 2004;10:193–6.
60. Evans C, Kheradmand F, Corry D, Tuvim M, Densmore C, Waldrep C, et al. Gene therapy of mucus hypersecretion in experimental asthma. Chest. 2002;121(3 Suppl): 90S–91S.
61. Foster KA. A new wrinkle on pain relief: re-engineering clostridial neurotoxins for analgesics. Drug Discov Today. 2005;10:563–9.
62. Ford E, Cruttwell C, Nute E, Chaddock J, Barnes P, Sutton J, et al. Inhibition of mucin secretion from A549 cells using a re-targeted clostridial endopeptidase. Proc Am Thor Soc. 2005;2:A219.
63. Burgel PR, Nadel JA. Roles of epidermal growth factor receptor activation in epithelial cell repair and mucin production in airway epithelium. Thorax. 2004;59:992–6.
64. O'Donnell RA, Richter A, Ward J, Angco G, Mehta A, Rousseau K, et al. Expression of ErbB receptors and mucins in the airways of long term current smokers. Thorax. 2004;59:1032–40.
65. Longphre M, Li D, Li J, Matovinovic E, Gallup M, Samet JM, et al. Lung mucin production is stimulated by the air pollutant residual oil fly ash. Toxicol Appl Pharmacol. 2000; 162:86–92.
66. Shim JJ, Dabbagh K, Takeyama K, Burgel PR, Dao-Pick TP, Ueki IF, et al. Suplatast tosilate inhibits goblet-cell metaplasia of airway epithelium in sensitized mice. J Allergy Clin Immunol. 2000;105:739–45.
67. Wang B, Lim DJ, Han J, Kim YS, Basbaum CB, Li JD. Novel cytoplasmic proteins of nontypeable Haemophilus influenzae up-regulate human MUC5AC mucin transcription via a positive p38 mitogen-activated protein kinase pathway and a negative phosphoinositide 3-kinase-Akt pathway. J Biol Chem. 2002;277:949–57.
68. Atherton HC, Jones G, Danahay H. IL-13-induced changes in the goblet cell density of human bronchial epithelial cell cultures: MAP kinase and phosphatidylinositol 3-kinase regulation. Am J Physiol Lung Cell Mol Physiol. 2003; 285:L730–L9.
69. Zhou Y, Shapiro M, Dong Q, Louahed J, Weiss C, Wan S, et al. A calcium-activated chloride channel blocker inhibits goblet cell metaplasia and mucus overproduction. Mucus Hypersecretion in Respiratory Disease. Chichester: Wiley, 2002. p. 150–65.
70. Nakanishi A, Morita S, Iwashita H, Sagiya Y, Ashida Y, Shirafuji H, et al. Role of gob-5 in mucus overproduction and airway hyperresponsiveness in asthma. Proc Natl Acad Sci U S A. 2001;98:5175–80.
71. Knight D. Talniflumate (Genaera). Curr Opin Investig Drugs. 2004;5:557–62.
72. Guzman K, Gray TE, Yoon JH, Nettesheim P. Quantitation of mucin RNA by PCR reveals induction of both MUC2 and MUC5AC mRNA levels by retinoids. Am J Physiol. 1996;271:L1023–8.
73. Yoon JH, Gray T, Guzman K, Koo JS, Nettesheim P. Regulation of the secretory phenotype of human airway epithelium by retinoic acid, triiodothyronine, and extracellular matrix. Am J Respir Cell Mol Biol. 1997;16:724–31.
74. Koo JS, Jetten AM, Belloni P, Yoon JH, Kim YD, Nettesheim P. Role of retinoid receptors in the regulation of mucin gene expression by retinoic acid in human tracheobronchial epithelial cells. Biochem J. 1999;338:351–7.
75. Apfel C, Bauer F, Crettaz M, Forni L, Kamber M, Kaufmann F, et al. A retinoic acid receptor alpha antagonist selectively counteracts retinoic acid effects. Proc Natl Acad Sci U S A. 1992;89:7129–33.
76. Bhattacharyya SN, Manna B, Smiley R, Ashbaugh P, Coutinho R, Kaufman B. Smoke-induced inhalation injury: effects of retinoic acid and antisense oligodeoxynucleotide on stability and differentiated state of the mucociliary epithelium. Inflammation. 1998;22:203–14.
77. Tesfaigzi Y, Fischer MJ, Martin AJ, Seagrave J. Bcl-2 in LPS- and allergen-induced hyperplastic mucous cells in airway epithelia of Brown Norway rats. Am J Physiol Lung Cell Mol Physiol. 2000;279:L1210–17.
78. Kellerman DJ. P2Y(2) receptor agonists: a new class of medication targeted at improved mucociliary clearance. Chest. 2002;121(5 Suppl): 201S–05S.
79. Roger P, Gascard JP, Bara J, de Montpreville VT, Yeadon M, Brink C. ATP induced MUC5AC release from human airways in vitro. Mediators Inflamm. 2000;9:277–84.
80. Jackson AD. Airway goblet-cell mucus secretion. Trends Pharmacol Sci. 2001;22:39–45.

Attorney Docket No. 5051-451IP PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re: Li et al.
Serial No.: 09/914,020
Filed: December 31, 2001
For: *Methods and Compositions for Altering Mucus Secretion*

Confirmation No.: 8515
Art Unit: 1633
Examiner: J. Epps-Ford

Date: June 7, 2006

Mailstop Amendment
Commissioner for Patents
PO Box 1450
Alexandria, VA 22313-1450

ATTACHMENT D

Agarwal et al. "*MARCKS Related Peptide Improves Airway Obstruction Related to Mucus Secretion in a Mouse Model of Asthma,*" Proceedings of the American Thoracic Society, Vol. 3, page A713, 2006.

Collagen I Content in Fatal Asthma

L.F. Silva[1], B.B. Araujo[1], H.A.P. Gomes[1], T. Mauad[1], J.H. Lindeman[2], A. Mulder[2], P.H.N. Saldiva[1], M. Dolhnikoff. [1]Department of Pathology – University of Sao Paulo Brazil; [2]Leiden University Medical Center Netherlands. Email: burns@usp.br

RATIONALE: It has been suggested that the small airways are involved in the mechanism of airway remodeling in asthma and may be responsible for the persistent airway obstruction and decline in lung function observed in some asthmatics. Several components of the airway extracellular matrix (ECM) may be altered in asthma, leading to increased thickness of the airway wall. We have recently observed that the total collagen is increased in small airways (SA) and peribronchiolar alveoli(PA) in fatal asthma (FA), and that the collagen III is decreased in the outer layer of SA. We now hypothesize that if the collagen I is increased, a type switch of collagen may be involved in airway remodeling in FA.

AIM: To determine the content of collagen I (ColI) in airways and alveolar parenchyma in FA and compare to non-asthmatic controls (CTR).

METHODS: We studied the distal lung of 19 patients that died of FA and 10 controls. Using immunohistochemistry and image analyses we measured the content of ColI in the outer layer (OL), smooth muscle (SM) and inner layer (IL) of large and small airways and in PA and distal alveoli (DA). Values were expressed as collagen area(CA)/BM length in OL and IL, as CA/SM area in SM, and as CA/septum length in the alveoli.

RESULTS: There was a significant increase in the content of ColI in the outer and inner layers of SA in asthmatics compared to controls [OL: FA=12.60(7.08-19.13 CTR= 7.07(2.78-19.91) p=0.001; IL: FA=4.29(2.11-6.85), CTR=2.41(0.66-3.84) p<0.001; PA: FA=1.65(0.62-2.93), CTR=0.58(0.17-1.26) p<0.001]. No significant differences were observed in large airways, in the SM of SA and in DA between groups.

CONCLUSIONS: The results show that the SA are involved in the mechanism of airway remodeling in FA with increased content of collagen I. These changes are extended to the peribronchiolar but not to distal parenchyma.

This Abstract is Funded by: FAPESP, CNPq, LIM/HCFMUSP.

Evaluating Airway Responsiveness to Multiple Bronchoconstricting Agonists in Strains of Mice – BALB/c and C57Bl/6 – Following Chronic Allergen Exposure

J.A. Hirota[1], J.N. Warrie[1], M.D. Inman[1]. [1]McMaster University, Hamilton, ON, Canada. Email: hirotaja@mcmaster.ca

Introduction: We have previously observed sustained airway hyperresponsiveness (AHR) and increases in contractile element content in BALB/c mice following chronic exposure to ovalbumin (OVA). These observations were absent from C57Bl/6 mice. To investigate the involvement of increased contractile elements in sustained AHR, we examined airway responsiveness to acetylcholine, 5-hydroxytryptamine, and leukotriene D4 in thin lung slices from BALB/c, and C57Bl/6 mice following chronic allergen exposure.

Methods: All mice were sensitized with intraperitoneal OVA on days 1 and 11, and intranasal OVA on day 11. On day 20, mice were then subjected to a chronic period of intranasal allergen exposure. Airway morphometry of was performed. Airway physiology measurements were made using lung slices (120um) of right lobe. Agonists were administered in a dose response fashion via a constant perfusion apparatus.

Results: BALB/c mice, but not C57Bl/6, showed significant increases in contractile element content in the airways ($p<0.05$). BALB/c mice, but not C57Bl/6, demonstrated a significant increase in response to 5-hydroxytryptamine following chronic allergen exposure ($p<0.05$). C57Bl/6 mice, but not BALB/c, demonstrated a significant increase in response to acetylcholine ($p<0.05$).

Conclusion: A quantitative increase of contractile elements in BALB/c mice is associated with lung slice assessed AHR to 5-hydroxytryptamine alone, suggesting that agonist specific signal transduction pathways may be altered following chronic allergen exposure. The absence of allergen induced quantitative increases of contractile elements in C57Bl/6 mice despite AHR to acetylcholine also supports this hypothesis. Further exploration into the effect of chronic allergen exposure on airway smooth muscle signal transduction pathways is warranted.

This Abstract is Funded by: Canadian Institutes of Health Research.

Effects of Roflumilast Treatment in Mice Following Chronic Allergen Challenge

R. Ellis[1], J. Wattie[1], M.D. Inman[1]. [1]McMaster University, Hamilton, Canada.

Introduction The pathogenesis of airway remodeling in asthma is poorly understood but it has been suggested that ongoing inflammatory and repair processes may be contributing factors. Inhaled corticosteroids and β_2-agonists are the mainstay in asthma therapy. Other agents may be required to either prevent or functionally reverse airway remodeling. Roflumilast, an investigational, oral, once-daily phosphodiesterase 4 (PDE4) inhibitor, has shown anti-inflammatory effects in patients with asthma and COPD. Thus, we investigated the effects of roflumilast on airway function and structure in a model of chronic allergic asthma.

Methods BALB/c mice were sensitized with intraperitoneal ovalbumin (OVA) given on Days 1 and 11, and intranasal (i.n.) OVA on Day 11. Animals were then exposed to i.n. OVA on 2 consecutive days during 6 challenge periods, each separated by 12 days. During each challenge period, roflumilast (5 mg/kg) or placebo was given once daily by gavage for 4 days (1 day prior, 2 days during, 1 day after challenge). Control mice were challenged with i.n. saline. Following a 4-week recovery after the last OVA exposure, airway hyperresponsiveness (AHR) to i.v. methacholine and airway morphometry were studied.

Results Non-treated mice chronically exposed to OVA allergen developed sustained AHR and structural airway changes consistent with aspects of airway remodeling as compared to saline-challenged controls. Treatment with roflumilast completely prevented the development of AHR as well as significantly reduced smooth muscle tissue and the number of mucin-containing goblet cells in the airways. Increased collagen detected in OVA-challenged mice was not altered by roflumilast.

Conclusion This study showed that oral roflumilast prevented functional and structural airway changes in this mouse model of chronic exposure to allergen. The demonstrated effects of roflumilast may translate into clinical benefits of anti-inflammatory treatment for patients with asthma.

This Abstract is Funded by: ALTANA Pharma AG, Germany.

Characterization of Early Phase Pulmonary Responses and Airway Hyperresponsiveness in Sheep Chronically Challenged with House Dust Mite

E. Koumoundouros[1,2], R.J. Bischof[1], E.N. Meeusen[1], I.M. Marcels[2], K.J. Snibson[1]. [1]Centre for Animal Biotechnology; [2]Department of Electrical Engineering, University of Melbourne, VIC, Australia. Email: emmanuel@unimelb.edu.au

Rationale: House dust mite (HDM) is a relevant allergen that is known to be involved in both the cause and exacerbation of chronic asthma. We have previously shown that repeated challenges of HDM to sheep lungs results in significant remodeling of the airways. We now assess lung function in chronically challenged sheep.

Methods: House dust mite was administered as either an aerosol or infusion on a weekly basis over a six month period and lung mechanics was assessed on a monthly basis. Airway responsiveness was determined as the dose of inhaled carbachol that increased airway resistance by 200% above baseline.

Results: An analysis of lung function data from all HDM-challenged sheep revealed that baseline pulmonary resistance increased at an average of two-fold over the repeated challenge period, and this increase could not be reversed with bronchodilator aerosols. Data from aerosol challenges with HDM clearly show that sheep segregate into two groups in terms of their early asthmatic responses to HDM. In particular, there were significant increases in airway resistance in the first hour post HDM challenge in a proportion (7/12) of HDM-challenged sheep at all the monthly time-points assessed. Non-responding HDM-challenged sheep (n=5), and all saline-challenged sheep (control, n=6) did not have increases in airway resistance after challenge during this period. The magnitude of the early asthmatic response progressively increased in HDM responder sheep. Both responder and non-responder sheep developed significant BAL eosinophilia and bronchial hyperresponsiveness during the challenges.

Conclusions: These results show that the sheep model is an appropriate animal model to examine the diversity of allergic responses and functional relationships involved in chronic asthma.

This Abstract is Funded by: None.

MARCKS Related Peptide Improves Airway Obstruction Related to Mucus Secretion in a Mouse Model of Asthma

A. Agrawal[1,2], R. Subramanian[3], K.B. Adler[4], A. Ram[3], B. Ghosh[3], M. Fahim[3], B.F. Dickey[5]. [1]Baylor College of Medicine, Houston, TX; [2]Vallabhbhai Patel Chest Institute, Delhi, India; [3]Institute of Genomic and Integrative Biology, Delhi, India; [4]North Carolina State University, Raleigh, NC; [5]MD Anderson Cancer Center, Houston, TX. Email: aagrawal@bcm.tmc.edu

Allergic asthma is associated with airway epithelial cell mucous metaplasia and mucus hypersecretion, but the consequences of mucus hypersecretion on airway function are unclear. Recently, a peptide derived from the MARCKS protein N-terminal sequence (MANS), was shown to inhibit airway mucus secretion (M. Singer et al, 2004). We studied the effect of intranasal pre-treatment with this peptide on airway function during challenge with the mucus secretagogues methacholine (MCh) and ATP in mice with allergen induced mucous cell metaplasia. We found that pretreatment with MANS but not a control peptide (RNS), resulted in inhibition of the fall in specific airway conductance (SGaw) induced by MCh, a secretagogue that also causes smooth muscle constriction (mean±SE; baseline 1.15±0.06; MANS/MCh 0.82±0.05; RNS/MCh 0.55±0.05 (cm $H_2O.s)^{-1}$). Additional challenge with ATP, a mucus secretagogue that relaxes airway smooth muscle in mice, resulted in normalization of SGaw in the MANS-pretreated group but not the control group (MANS/MCh/ATP, 1.1±0.1; RNS/MCh/ATP, 0.8±0.05). The protective effects of MANS peptide were sustained in mice with increased airway hyperresponsiveness due to repeated allergen challenges (daily for 3 days), but the magnitude of the fall in SGaw increased (baseline 1.1±0.08; MANS/MCh, 0.65±0.06; RNS/MCh, 0.47±0.03). In all instances, SGaw in the MANS-pretreated group was about 35% higher than control. This is equivalent to 20% change in FEV1 and is biologically significant. In summary, therapy targeting mucin secretion improved airflow obstruction in an animal model of asthma comparable to accepted therapies in humans.

This Abstract is Funded by: NIH R01HL072984, R37HL36982.

Compensatory Lung Growth after Pneumonectomy Is Partially Achieved by Formation of New Alveoli in Mice

H. Fehrenbach[1], V. Michl[1], R. Voswinckel[2], A. Fehrenbach[1], W. Seeger[2], H.J. Gundersen[3], J.R. Nyengaard[3]. [1]Clinical Research Group "Chronic Airway Diseases", Philipps-University, Marburg, Germany; [2]University of Giessen Lung Center, Giessen, Germany; [3]Stereology & Electron Microscopy Research Laboratory, University of Aarhus, Aarhus, Denmark. Email: fehrenba@med.uni-marburg.de

After left-sided pneumonectomy (PNX), the remaining right lung completely restores alveolar surface area and septal volume of the excised left lung by day 21 (Voswinckel *et al* 2004, *Eur Respir J* 24:524-32). To investigate if structural restoration of the gas exchange region was accomplished by growth of existing alveoli or formation of new alveoli, numbers of alveoli were determined by design-based stereology. PNX was performed in C57BL/6 mice (14-16 wks). Lungs were excised at day 7 (n=6) or day 21 (n=5) after PNX. Lungs of PNX and control mice (n=10) were fixed via the trachea (20cm H_2O). Tissue blocks were systematic uniform random sampled according to a fractionator design. The disector principle was used to estimate alveolar numbers (Hyde *et al* 2005, *Anat Rec* 277A:216-26). Means± sd are given. A progressive increase in the number of alveoli (N alv) of the right lung was observed in PNX mice (Table 1). By day 21, N alv per right lung was significantly increased by 33% in PNX compared with right lungs of controls. Total N alv of control mice was still significantly higher than N alv of PNX right lungs. However, whereas in controls the right lung accounted for 60±12% of total N alv, the right lung of PNX mice (day 21) achieved 82±13% of mean total N alv of control mice. We conclude that structural restoration of the gas exchange region after PNX in mice is partially achieved by formation of new alveoli.

Table 1 - Numbers of Alveoli (N alv)

Group	days post PNX	N alv (total)	N alv (right lung)
Control	—	$11.6\pm1.4\times10^8$	$6.9\pm1.6\times10^8$
PNX	07	$8.7\pm2.3\times10^{8*}$	$8.7\pm2.3\times10^8$
PNX	21	$9.2\pm1.5\times10^{8*}$	$9.2\pm1.5\times10^{8*}$

* $p<0.05$ versus control (ANOVA)

This Abstract is Funded by: BMBF (FKZ 01GC0103).